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Vaccines for *Yersinia pestis* *Final Report*

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FINAL REPORT

**For the Department of National Defense,
Defense Research Establishement Suffield**

Contract #W7702-5-R527/001/EDM

Vaccines for *Yersinia pestis*

VIDO

VETERINARY
INFECTIOUS
DISEASE
ORGANIZATION

102-01448/

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Vaccines for *Yersinia pestis*

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EXECUTIVE SUMMARY

The objective of this research contract was to construct prototype vectored vaccines producing *Yersinia pestis* antigens and to test their ability to protect mice against experimental challenge. In addition, the work described in Appendices A and B to Annex A dealt with the potential of intranasally-delivered conventional *Y. pestis* antigens formulated in liposomes to protect against pneumonic plague. The genes coding for the pH6 and V antigens were obtained from researchers in the United States. They were expressed at high levels in *Escherichia coli* in order to produce subunit antigens needed for immunological assays as well as to raise specific polyclonal antiserum in rabbits. The gene coding for the V antigen genes were inserted into the E3 region of human adenovirus while both the pH6 operon as well as the V antigen was inserted into live-attenuated *E. coli* vaccine strains. These prototype vaccine strains were fully characterized and delivered to the Defense Research Establishment Suffield for vaccine testing. Immunization of mice at mucosal sites did not result in the induction of protective immunity.

A second set of experiments described in Annex A as well as Appendices A and B dealt with the feasibility of reformulating the existing whole cell plague vaccine into liposomes for intranasal immunization. The results demonstrated that incorporation could be efficiently achieved and that excellent systemic and mucosal immune responses could be obtained following immunization with lower antigen dose than is currently used. These vaccine formulations were shown to protect mice against pneumonic plague in experimental challenge studies.

The recombinant V antigen was also tested for its protective capacity in mice following intranasal immunization. In contrast to the results obtained by others using V antigen delivered by the subcutaneous route, no protection was observed despite an excellent antibody response. Further work will be required to determine the reason(s) for this lack of efficacy.

Research Report – Annex A

Development of A Mucosal Vaccine Vector for *Yersinia pestis*

I. Objectives

The objective of this research was to develop mucosal vaccines for the prevention of pneumonic plague. This included the following specific aims as outlined in Annex of the contract.

PART I. Vectored Vaccines for *Y. pestis*

1. Obtain genes coding for the pH6 and V antigens of *Y. pestis*. If not available, the genes will be cloned using PCR technology.
2. Construct an *E. coli* vaccine vector.
3. Construct a human adenovirus vaccine vector.
4. Develop reagents and assays to test immune responses.
5. Test immune responses *in vivo*.
6. Deliver vectors to DRES.
7. Attend tri-national meeting on plague vaccine research at United States Army Research Institute for infectious diseases (USAMRIID), Frederick, Maryland, December 1998.

PART II. *Yersinia pestis* Liposome-based Vaccines

8. Submit and obtain approval for the CCAC-approved animal care committee at the University of Saskatchewan for studies outlined below in (9) and (10).
9. Determine if using a dose of 5 μg of *Y. pestis* bacterin formulated in liposomes is protective against intranasal challenge.
10. Determine whether a single dose of the vaccine can protect against intranasal challenge.

Research Report – Annex A, Part I

Vectored Vaccines for *Yersinia pestis*

I. Objectives

PART I. Vectored Vaccines for *Y. pestis*

1. Obtain genes coding for the pH6 and V antigens of *Y. pestis*. If not available, the genes will be cloned using PCR technology.
2. Construct an *E. coli* vaccine vector.
3. Construct a human adenovirus vaccine vector.
4. Develop reagents and assays to test immune responses.
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6. Deliver vectors to DRES.
7. Attend tri-national meeting on plague vaccine research at United States Army Research Institute for infectious diseases (USAMRIID), Frederick, Maryland, December 1998.

II. Introduction

Current vaccination against *Yersinia pestis* relies upon intramuscular immunization with a formalin-killed whole cell bacterin vaccine. However, evidence suggests that while the vaccine protects against bubonic plague, it is not fully protective against pneumonic plague. Thus, there is a need for an improved vaccine which will protect against respiratory challenge, the most likely route of infection envisaged in biological warfare. Current molecular biology techniques enable protective antigens to be genetically cloned and expressed in vectors, which allow targeting of the antigen to the appropriate immunological compartment e.g. the gut and bronchial-associated lymphoid tissues. This is of fundamental importance if mucosal immunity in the lungs is required. This technology also allows the development of defined vaccines consisting of protective antigens but void of irrelevant and often undesirable components such as lipopolysaccharide. The vectors often replicate *in vivo* and these "living" vectors have evolved mechanisms which prevent immediate clearing by the innate immunity of the host; this persistence can induce immunity that is more effective and of longer duration than conventional vaccines. Furthermore, the vectors often have the capacity to invade cells resulting in more potent cell-mediated immunity. Recombinant vaccines can be safer to produce and are subject to much less batch-variation than crude whole cell vaccines. The Veterinary Infectious Disease Organization has developed a number of technologies for the use of a number of mucosal vaccine vectors, including *E. coli* and Adenoviruses, and the main objective of this contract was to develop a mucosal vaccine vector for the delivery of *Yersinia* antigens using these vectors.

Two putative protective components, the pH6 antigen and the V antigen, were chosen for this study. The pH6 antigen is a pilus adhesion expressed at the cell surface at pH < 6.7. It is produced by *Y. pestis* within macrophages and is thought to play a role in attachment. A protective role for pH6 antigen has not been established, but mutants of *Y. pestis*, which do not produce the antigen, have been shown to have reduced virulence. In contrast, the V antigen is a released protein and a potent immunomodulator, which perhaps functions *in vivo* as an aggressin. Recently, recombinant V antigen has been shown to be protective against both bubonic and pneumonic plague. Enteroinvasive *Escherichia coli* and human adenovirus serotype 5 were chosen as delivery systems for these *Yersinia* antigens. Adenovirus has already been used to safely immunize US military recruits and recombinant adenovirus 5 has been shown to induce mucosal immunity for a variety of viral antigens. Attenuated invasive bacteria have been widely used as delivery systems.

III. Materials and Methods

Strains and plasmids For DNA manipulations *E. coli* DH5 (*φ80 lacZΔM15 endA1 hsdR17*(r_K^- m_K^+)*supE44 thi-1λ gyrA96 relA1Δ(lacZYA-argF) U169*) and DH5αF' (/F' *lac*^{q} *proAB*$^-$ *LacZ*]*M15 Tn5* (Km^r)) were used. *E. coli* BL21 DE3 (F^- *ompT hsdS*$_B$ (r_B^- m_B^-) with DE3, a λ prophage carrying the T7 RNA polymerase gene) was used for high level expression of recombinant antigens. *E. coli* LCB273 (*aroA273::Tn10*) was obtained from the *E. coli* Genetic Stock Center. Enteroinvasive *E. coli* EI-4 serotype O28ac H^- Lac^- was provided by Myron Levine (Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland 21201). Plasmids pJITZ and pDG5 containing *Yersinia* genes were obtained from Dr.'s Susan Straley (University of Kentucky) and Luther Lindler (NIH). Plasmids pFG173 and pFG144.15SV40 for the Human Adenovirus 5 constructs were obtained from Frank Graham , Department of Biology and Pathology, McMaster University, Hamilton, Ontario. *Yersinia pestis* strain GB was provided to DRES by the Chemical and Biological Defense Establishment, Porton Down, UK.

Transfection and growth of adenovirus E293 cells were grown in Eagle's minimum essential medium (MEM) with 5% fetal bovine serum (FBS). Cells were transfected with plasmids using the reagent lipofectamine (Gibco BRL, Gaithersburg, MD U.S.A) according to the Manufacturer's instructions. Cells were observed for cytopathic effect which normally occurred after 5 days. Positives were plaque purified and verified to contain the recombinant gene by PCR. Adenovirus was propagated in E293 cells, titred and stored at -70°C before challenging mice.

Radiolabelling and Immunoprecipitation About 70 – 80% confluent HELA cells and MDBK cells were infected at a MOI of 10. After virus absorption for 60 min, the cells were incubated in MEM containing 5% FBS for 6 h. The cells were incubated for 1 h in methionine-free MEM before adding [^{35}S] methionine for 12 and 24 h. The cells were harvested, lysed and proteins immunoprecipitated and analyzed by SDS-PAGE as described previously (Tikoo *et al.*, 1993).

DNA manipulations. Constructs were made using standard Molecular Biology Techniques (Sambrook, *et al.*, 1989). PCR was performed using Pfu polymerase (Strategene, La Jolla, CA, USA) according to the manufacturers instructions using 20 – 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min; sufficient cycles were used until the correct PCR product could be visualized on an agarose gel.

Western blot and SDS-PAGE analysis Bacterial lysates or protein fractions were analyzed on dodecyl sulfate-polyacrylamide (SDS-PAGE) gels (Laemmli, 1970) and stained with Coomassie blue. For

Western-blot analysis, proteins electrophoretically separated by SDS-PAGE were transferred to nitrocellulose using a Bio-Rad Transblot apparatus (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Membranes were blocked in 2% bovine serum albumin (BSA) in PBS overnight at 4°C. Membranes were probed with antisera diluted 1/1000 in PBS with 2% BSA for 1 h at room temperature and washed 3 times with PBS. The membranes were then incubated with goat anti-rabbit conjugated with alkaline phosphatase for 1 h and extensively washed before developing with nitro blue tetrazolium and bromo-chloro-indolyl phosphate.

P1 Transduction of Enteroinvasive *E. coli* This was carried out as described by Miller (1992). Briefly, a P1 lysogen of strain LCB273 was prepared and a heat-induced lysate was used to transduce strain EI-4. EI-4 transductants containing Tn10 insertions in *aroA* were selected for by tetracyline resistance conferred by Tn10. The aroA$^-$ phenotype was confirmed by the ability to grow on minimal medium only if supplemented by aromatic amino acids. Insertion of Tn10 in the *aroA* locus was also confirmed by PCR analysis.

Production of recombinant antigens and antisera BL21DE3 containing recombinant plasmid was grown to an A_{600} of 0.7 in 500 ml of LB medium and induced by the addition of IPTG to 0.2 mM and incubated with shaking for further 2 h.. Recombinant V antigen was expressed as a soluble protein with a six amino acid histidine tag and was purified as follows: Bacteria were harvested by centrifugation and resuspended in 15 ml of sonication buffer: 20 mM TrisHCl pH 7.8, 500 mM NaCl, 5 mM imidazol, 1 mM PMSF. Cells were disrupted by sonication on ice until of low viscosity. The lysate was mixed with 4ml of a 50% slurry of nickel chelated NTA resin (Qiagen, Chatsworth, CA 91311, USA) equilibrated with sonication buffer, and incubated for 1 h at 4°C with gentle mixing. The slurry was loaded into a 1 cm diameter column and allowed to drain at a flow rate of 0.5 ml/min. The column was washed with 6 volumes of sonication buffer followed by 20 mM Tris-HCl pH 7.8, 500 mM NaCl, 20 mM imidazol. The antigen was eluted with 12 ml of 20 mM Tris-HCl pH 7.8, 500 mM NaCl, 500 mM imidazol and dialyzed against PBS and concentrated. The pH 6 antigen, which was expressed as inclusion bodies, was prepared as follows: Bacteria were harvested by centrifugation and resuspended in 4 ml of 25 % sucrose 50 mM Tris-HCl pH 8.0 and freeze thawed. Lysozyme (1 ml of 10 mg/ml) was added followed by incubation at 4°C for 15 min. This was added to 30 ml of detergent mi x: 100 mM Tris-HCl pH 8.0, 150 mM NaCl, 25 mM EDTA, 1% Deoxycholic acid, 1% NP-40, 1% Triton X-100. This was sonicated until viscosity was reduced. The detergent extract was centrifugated at 12000 g for 20 min and washed twice with PBS. New Zealand white rabbits were immunized with subcutaneously

with 100 μg of recombinant protein mixed with an equal volume of complete Freunds adjuvant. After 21 days rabbits were boosted with an equal dose with incomplete Freunds adjuvant and bled 15 days latter.

Mouse protection test Female mice at 6 weeks of age (C57B1/6) were immunized in groups of 12. Mice were immunized with adenovirus intranasally; the mice were first anesthetized by metofane inhalation and adenovirus (a single dose of 10^7 pfu in MEM) was slowly applied to the nostrils. *E. coli* (5×10^9 cfu in 100 μl of PBS) was given orally and boosted after 21 days; mice were deprived of water for 4h and gently encouraged to drink the bacterial suspension from a disposable tip. Bacteria were first grown to mid exponential phase in LB medium, harvested and resuspended in PBS and administered to the mice on the same day. Thirty one days after immunization all groups of mice were anesthetized, and challenged intranasally with 25 μl containing 6100 cfu (10 MLD_{50}) of *Y. pestis*.

IV. Results

1. Source of genes

The genes coding for the pH6 – and V antigens were not obtained by DRES from USAMRIID and therefore they were obtained directly from Dr. Luther Lindler and Susan Straley by VIDO. In addition to this, we isolated the genes coding for the V antigen and pH6 antigen by PCR from a killed *Yersinia pestis* vaccine obtained from DRES. This killed vaccine contained sufficient nucleic acid to obtain an alternative source of the wild type genes.

2. Development of reagents and assays to test immune responses

Before constructing *E.coli* and adenoviral delivery systems for the V and pH6 antigen, it was necessary first to raise antisera to these antigens so that their expression in the delivery systems could be monitored. For this purpose, the structural genes for the V antigen and the pH6 antigen were PCR amplified and cloned into an expression vector. The gene for the pH6 subunit, with the leader sequence omitted, was PCR amplified from the *psa* operon contained within the plasmid pDG1 (Lindler *et al.*, 1990). The structural geneV antigen, which does not contain a leader sequence, was PCR amplified using plasmid pJIT7 (Perry *et al.*, 1986) as a template. To amplify the V antigen structural gene, the primers CCGAACGG*CATATG*ATTAGAGCCTACGAAC and CCG*GGATCC*TCATTTACCAGACGTGTCATC were used and the primers CCGTTCGG*CATATG*TCTACTGTCATTAACT and GGC*GGATCC*TTAAAATACATACTCTTCAACACGC were used to amplify the pH6 subunit gene; letters in bold indicate sequence with homology to *Yersinia* genes and italicized sequence indicate restriction sites for the enzymes *NdeI* and *BamHI*. PCR products were cleaved with *NdeI* and *BamHI* and cloned into the expression vector pET15b (Novagen, Madison, WI, U.S.A.) cut with the same enzymes. Constructs were sequenced to verify cloning of the correct products; plasmid containing V antigen sequence was designated pETV and plasmid containing pH6 subunit sequence was designated pET6. Recombinant proteins are expressed from the T7 promoter and as fusion proteins fused to a six amino acid histidine tag at the amino-terminal end. *E. coli* strain BL21(DE3), which expresses T7 polymerase, was transformed with the constructs to allow expression of the recombinant proteins. Preliminary experiments indicated that the pH6 subunit was expressed as an insoluble protein. Therefore, aggregates were prepared to conveniently immunize rabbits. On the other hand, the V antigen, which is a very soluble protein was expressed in the cytoplasm and was purified by virtue of the

histidine tag. Figure 1 shows an SDS-PAGE gel of the pH6 aggregate and the purified V antigen and Figure 2 shows a Western blot after antisera was raised in rabbits.

3. Construction of an *E. coli* vectored vaccine

Development of *E. coli* delivery system. A restriction map of the pH6 antigen is shown in Fig. 3 . The subunit of the pH6 antigen is encoded for by *psaA* while the other genes are required for the assembly of the subunit into pili on the surface of the bacteria. Pili antigens usually require quaternary structure for protection and pilin subunits are not by themselves protective. Therefore, the entire operon, containing all the accessory genes, was subcloned as a 6.5 kb *KpnI /ClaI* fragment from pDG5 into the vector pIC20R (Marsh *et al.*, 1984) to give pPH6. *E. coli* harbouring pPH6 was grown to saturation in LB medium and whole cells were analyzed in Western blots. Blots probed with anti-pH6 antigen demonstrated a ladder effect indicating the assembly of pili on cell surface (Fig. 4) indicating that this construct could serve to express relevant *Yersinia* antigen in *E. coli*. The gene for the V antigen, *lcrV* is similarly part of an operon (Fig. 5), which encodes in addition the genes for *lcrG* and *lcrH*. The functions of the G and H proteins are not known but may play a role in the regulation of YOP protein synthesis. A 2 kb *AccI/AvaII* fragment from pJIT7, which contained the *lcrGVH* operon, was cloned into the *SmaI* site of pTZ19r to give the plasmid pVOP. However, expression of the V antigen from pVOP in *E. coli* was rather poor perhaps because of the complex regulatory mechanisms that occur in *Yersinia*. Therefore, it was decided to express the V antigen from two *E. coli* promoters: namely, that for *GroE* and that for *NirB*. The *GroE* promoter directs the synthesis of a heat shock protein, whereas the *NirB* promoter directs the synthesis of an operon which includes the nitrate reductase gene. Both these inducible promoters have been used to express heterologous antigen in *Salmonella* (Everest *et al.*,1995 and McSorley *et al.*, 1997) and are upregulated in response to the harsh intracellular environment *in vivo*. The *GroE* promoter was PCR amplified from *E. coli* DNA using the primers GCGCAGACTCGAAATGTGAGGTGAATCAGG and CCGAACGG*CATATG*TAACTCTCCTTTGAGAAAGTC and the *NirB* promoter was amplified using the primers GCGCAGACTCATGCCGGACGGCACTAT and CCGAACCGC*CATATG*TGCCTCGATTTCTTTTCTATTACCGCC; *NdeI* sites are indicated in italics. The PCR products, 198 bp and 283 bp for *GroE* and *NirB* promoter regions respectively, were cloned into pIC20R (with the *NdeI* site removed) (Marsh *et al.*, 1984) cut with *EcoRV*. The orientation of the inserts were checked and clones were isolated where the *NdeI* site of the cloned promoters lay proximal to the *BamHI* site contained in the poly-cloning site of pIC20R. The 1kb *NdeI/BamHI* fragment from

pETV, containing the structural gene for the V antigen, was ligated into the above vectors cut with *NdeI* and *BamHI*. This resulted in the plasmids pICgroV and pICnirV where the gene for the V antigen was cloned downstream of the *GroE* and *NirB* promoters respectively. These promoters can be induced *in vitro* by growth at elevated temperatures. V antigen was expressed from both promoters and was greater after heat-shock induction (Fig. 6) Expression was highest from the *GroE* promoter but the *NirB* promoter has been reported to give better expression in *in vivo* conditions (Everest *et al.*, 1995 and McSorley *et al.*, 1997).

Attenuated aromatic acid-dependent (*aro*) *Salmonella typhimurium* strains have been well characterized as carriers for various heterologous antigens (Hormaeche *et al.*, 1994). It was therefore decided to develop an *aroA* attenuated enteroinvasive *E. coli* as a carrier for the *Yersinia* antigens. Strain EI-4 was attenuated by introducing a Tn10 insertion in *aroA* by P1 general transduction using a lysogen prepared from strain LCB273 (*aroA273*::Tn10). Tetracycline transductants of EI-4 were confirmed to have the *aroA* phenotype and a Tn10 insertion in *aroA*. *E. coli* EI-4 *aroA*::Tn10 was then transformed with the plasmids pPH6, pICgroV and pICnirV, and the expression of the *Yersinia* antigens was confirmed in Western blotts.

4. **Construction of adenovirus 5 expressing the V antigen**

We had originally proposed to express both the V- and pH6 antigens in human adenovirus. However, during the plague meeting in Fort Detrick, Maryland it became obvious that a subunit pH6 antigen would not be effective. Therefore, this gene was not inserted into adenovirus and rather we focused upon the V antigen. Since the *E. coli* vector expressed the pH6 antigen as an assembled pilus (see above) this was to be used in the vaccine trials. Adenovirus 5 (Ad5) was constructed with the structural gene for the V antigen cloned in a deleted E3 region downstream from the E3 region. Ad5 expressing pH6 antigen was not constructed as it is the quaternary stucture of pili antigens that is required for protection as discussed previously. The structural gene of the V antigen was PCR amplified using the primers CCGAA*GGATCC*ATGATTAGAGCCTACGAACA and CCGATGCT*GTCGAC*TCATTTACCAGACGTGTCATC; italicized sequence indicates *BamHI* and *SalI* restriction sites and sequence in bold indicates sequence complementary to *Yersinia* sequence. The PCR product was cut with *BamHI* and *SalI* and cloned into the E3 region of plasmid pFG144.15SV40 cut with *BamHI* and *XhoI* (which has compatible overhangs with *SalI* cuts). This vector contains a deleted E3 region with an early SV40 promoter downstream of the E3 promoter, followed by a *BamHI* and *XhoI* sites and a SV40 polyA region. E293 cells of low passage (>30) were transfected with the

E3/V construct together with pFG173 (which contains the remainder of the Ad5 genome in addition to regions of homology). Replicative recombinant Ad5 virus results from homologous recombination between the two plasmids. Five days after transfection, cytopathic effect was observed in some wells and these were transferred to fresh cells to confirm replicative virus. One isolate was plaque purified and verified to contain the structural gene for the V antigen in the E3 region by PCR analysis; this strain was designated Ad5V. Expression of the V antigen after Hela cells and MDBK cells were infected with Ad5V and wild type Ad5 was determined by immunoprecipitation (Fig. 7). Expression of V antigen was very poor although a faint band corresponding approximately to the correct molecular weight, was observed 24 h after infection of Hela cells, in addition there were some signs of low molecular weight products (>30,000 kD) which may be truncated versions. Bacterial antigen genes have not been expressed in adenovirus and several factors such as promoter, intron use, codon bias and elimination of putative excision recognition sites may have to be optimized further for high level expression.

5. **Immune responses following immunization**

Seven groups of mice were immunized as follows: group 1, Ad5 (wild type); group 2, Ad5V; group 3 UV inactivated Ad5V; group 4, *E. coli* EI-4 *aroA*; group 5, *E. coli* EI-4 *aroA* pPH6; group 6, *E. coli* EI-4 *aroA* pICgroV; and group 7, *E. coli* EI-4 *aroA* pICnirV. However, no groups showed any protection (Table 1). It is not known whether antibodies were produced to either of the *Yersinia* antigens indicating *in vivo* synthesis of pH6 or V antigen.

Recombinant V antigen. It was thought that the recombinant V antigen produced from pETV might be useful as a vaccine and purified recombinant V antigen was incorporated into lipsomes as indicated in the accompanying report "Intranasal immunization with liposome-formulated *Yersinia pestis* vaccine". However, mice immunized with V antigen incorporated into lipsomes were not protected (Fig. 8) although a high titre of anti-V IgG was detected in the serum. In addition, mice immunized with the V antigen liposomes had the highest titre of anti-V IgG in comparison to mice immunized with bacterin incorporated liposomes (Figure 8) although the later were significantly protected against challenge. This contradicts the findings of other groups who found protection to be correlated with antibody to V antigen (Leary *et al.*, 1995, Anderson *et al.*, 1996). It may also explain why none of adenovirus or *E. coli* delivery systems for V antigen protected against pneumonic challenge. A protective role for the pH6 antigen has still to be established but it is unknown whether antibody to the pH6 antigen was produced in animals immunized with *E. coli* pPH6.

6. **Delivery of vaccine vectors to DRES**

All *E. coli* strains as well as the vectored adenovirus isolate were delivered to DRES prior to conducting the mouse studies described above.

7. **Attend tri-national plague vaccine meeting, December 1998.**

The above meeting was attended by Dr. Andrew Potter (with Dr. B. Koumakakis and colleagues), who gave a presentation on the work described in this report.

V. References

Anderson G.W., Leary S.E.C., Williamson E.D., Titball R.W., Welkos S.L., Worsham P.L. and Friedlander A.M. (1996) Recombinant V antigen protects mice against pneumonic and bubonic plague caused by F1-capsule-positive and –negative strains of *Yersinia pestis*. *Infection and Immunity 64*, 4580-4585.

Everest P., Frankel G., Li J., Lund P., Chatfield S. and Dougan G. (1995) Expression of LacZ from *htrA*, *nirB* and *groE* promoters in a *Salmonella* vaccine strain: Influence of growth in mammalian cells. *FEMS Microbiology Letters 126*, 97-102.

Hormaech C.E., Khan C.M.A., Mastroeni P., Villarreal B., Dougan G., Roberts M. and Chatfield S.N. (1994) Salmonella vaccines: mechanisms of immunity and their use as carriers of recombinant antigens. pp. 119-153 In: Molecular and clinical aspects of bacterial vaccine development. Eds. Ala'Aldeen D.A.A. and Hormaeche C.E. John Wiley and Sons Ltd., England.

Imler J. (1995) Adenovirus vectors as recombinant viral vaccines. *Vaccine* 13, 1143-1151.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London) 227*, 680-685.

Leary S.E.C., Williamson E.D., Griffen K.F., Russell P., Eley S.M. and Titball R.W. (1995) Active immunization with recombinant V antigen from *Yersinia pestis* protects mice against plague. *Infection and Immunity 63*, 2854-2858.

Lindler L.E., Klempner M.S. and Straley S. (1990) *Yersinia pestis* pH 6 antigen: genetic, biochemical,and virulence characterization of a protein involved in the pathogenesis of bubonic plague. *Infection and Immunity 58*, 2569-2577.

Marsh J.L., Erfle M. and Wykes E.J. (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. *Gene 32*, 481-485.

McSorley J.S., Xu D. and Liew F.Y. (1997) Vaccine efficacy of *Salmonella* strains expressing glycoprotein 63 with different promoters. *Infection and Immunity 65*, 171-178.

Miller J.H. (1992) A short course in bacterial genetics. Cold Spring Harbor Laboratory Press. Cold Spring Harbor. U.S.A.

Perry R.D., Harmon P.A., Bowmer W.S and Straley S.C (1986) A low-Ca^{2+} response operon encodes the V antigen of *Yersinia pestis*. *Infection and Immunity 54*, 428-434.

Sambrook J., Fritsch E.F. and Maniatis T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory. Cold Spring Harbor. U.S.A.

Tikoo S.K., Zamb T.J., and Babiuk L.A. Analysis of bovine herpesvirus-1 glycoprotein gIV truncations and deletions expressed by recombinant vaccinia viruses (1993). *Journal of Virology 67*, 2103-2109.

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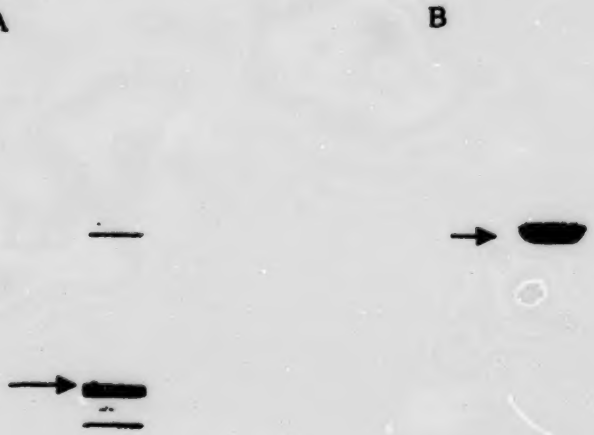

Figure 1. SDS-PAGE of recombinant pH6 and V antigens

A. SDS-PAGE gel of recombinant pH6 antigen preparation. The arrow points to the 15 kDa subunit of the pH6 antigen. An aggregate preparation (10 μg) was solubilized and analyzed on a 12% SDS-PAGE gel.

B. SDS-PAGE gel of affinity purified recombinant V antigen. The arrow points to the 37 kDa V antigen fused with a 6 amino acid histidine tag. The recombinant antigen was affinity purified as previously described and 10 μg was solubilized and analyzed on a 12 % SDS-PAGE gel.

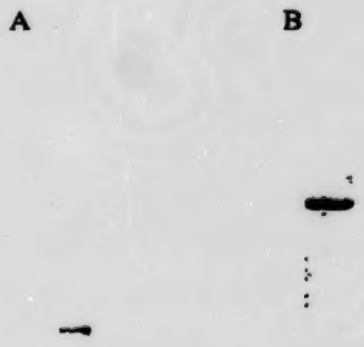

Figure 2. Western blots of recombinant pH6 and V antigens

A. Rabbits were immunized with the recombinant pH6 aggregate preparation and antisera was used to probe recombinant pH6 antigen and electrophoretically separated on a 12% gel. The single band observed corresponds with the 15kDa pH6 subunit.

B. Antiserum raised to the affinity purified V antigen was similarly used to probe recombinant V antigen electrophoretically separated. The single band observed corresponds with the 37 kDa V antigen.

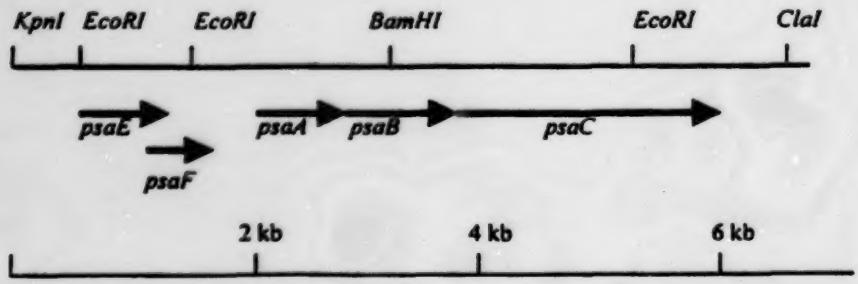

Figure 3. Restriction and functional map of the *Y. pestis* pH6 antigen operon.

Figure 4. Expression of pH6 antigen in *E. coli*

E. coli harbouring pPH6 (lane 1) was grown to saturation, harvested and resuspended in 1/10 culture volume of PBS. The suspension was resuspended in an equal volume of solubilization buffer and 20 µl was run on an SDS-PAGE gel, transferred to nitrocellulose and probed with anti-pH6. The *E. coli* strain without plasmid is shown in lane 2.

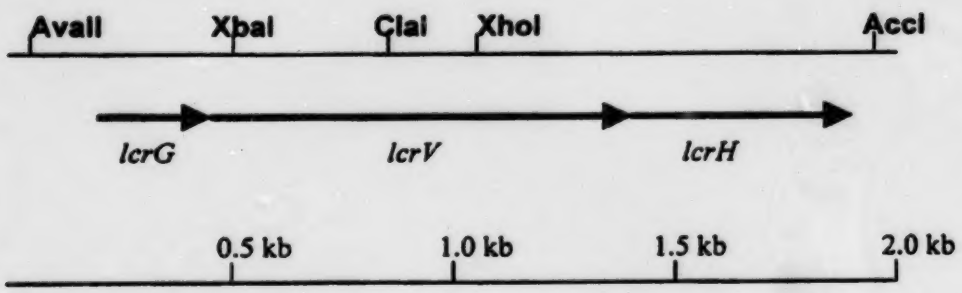

Figure 5. Restriction map and functional organization of the V operon.

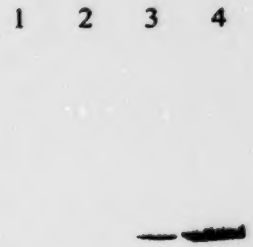

Figure 6. Expression of V antigen from *GroE* and *NirB* promoters

E. coli harbouring pICnirV (lanes 1 and 2) and pICgroV, were grown to mid exponential phase and incubated for a further hour at 37°C (lanes 1 and 3) or 42°C (lanes 2 and 4). *E. coli* was harvested and resuspended in 1/10 volume of PBS, and mixed with an equal volume of solubilizing buffer. The suspension (20 µl) was electrophoretically separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-V serum.

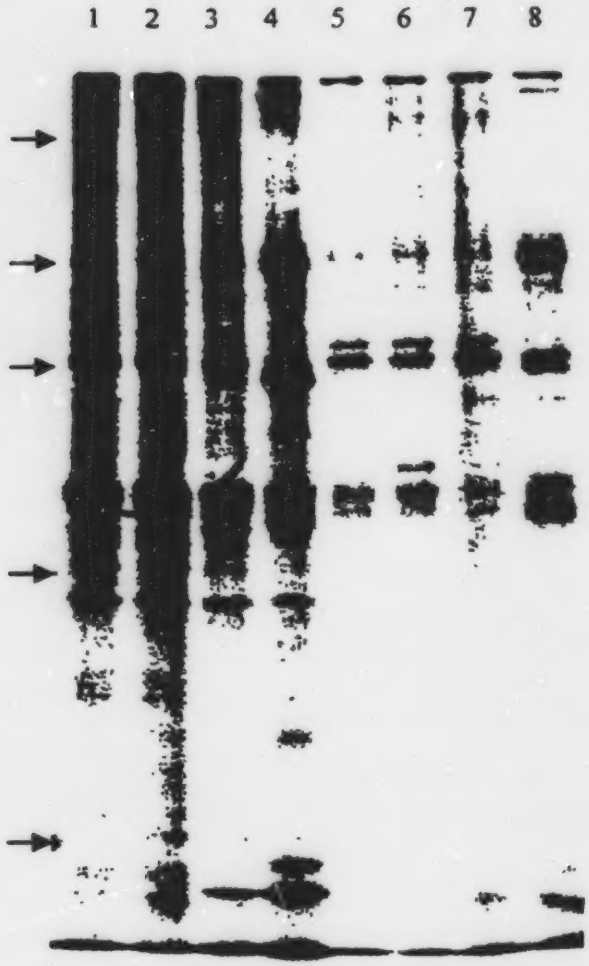

Figure 7. Immunoprecipitation of adenovirus infected cell lines with anti-V serum

Hela cells (lanes 1-4) and MDBK cells (lanes 5-8) were infected with wild type Ad5 (lanes 2 and 6) and recombinant Ad5V containing the V gene in E3 region (lanes 3,4,7 and 8). Lanes 1 and 5 are uninfected controls. After infection, cells were incubated with [^{35}S] methionine for 12h (lanes 2,3,6 and 7) and 24h (lanes 4 and 8). Arrows correspond to molecular weights of 200, 97, 69, 46 and 30 kDa.

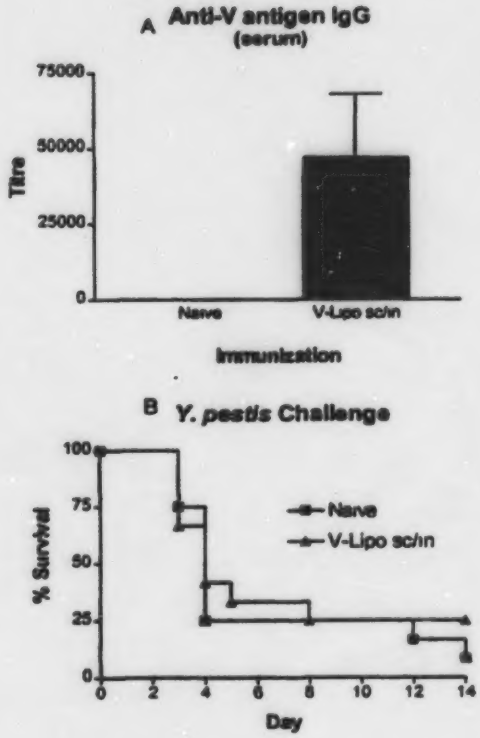

Figure 8. Humoral anti-V titres after immunization with V antigen incorporated into liposomes and mortality following *Y. pestis* challenge.

(A) Systemic humoral immune responses, and; (B) Protection against *Y. pestis* challenge. Mice were immunized with 10 μg of recombinant V antigen formulated in liposomes. Animals received a primary immunization with V antigen by the subcutaneous (s.c.) route and 21 days later were boosted intranasally (i.n.). The anti-V antigen IgG titres were determined in serum. Antibody titres are expressed as the mean titre ± SEM of 5 individual mice. Two weeks after the second immunization all animals were challenge with 10 LD_{50} of *Y. pestis*.

Table 1. Mortality of immunized mice after challenge with *Y. pestis*

Group	No. of deaths
1. Ad5 (wild type)	12/12
2. Ad5V	11/12
3. Ad5V (UV inactivated)	8/11
4. E1-4 *aroA*	5/11
5. EI-4 *aroA* pPH	9/12
6. EI-4 *aroA* pICgroV	9/11
7. EI-4 *aroA* pICnirV	12/12

Mortality was recorded 1 week after challenge with *Y.* pestis

Research Report- Annex A, Part II

***Yersinia pestis* Liposome-based Vaccines**

I. Introduction

The work of a previous contract was designed to assess whether the route of delivery and formulation of *Yersinia pestis* vaccine could alter the induction of systemic and mucosal immune responses, and whether these responses could protect animals form an intranasal challenge with *Y. pestis* (pneumonic plague model). The results from those experiments demonstrated that induction of mucosal immunity in the respiratory tract is efficiently induced by intranasal delivery of the vaccine. In addition, mice immunized by the intranasal route were better protected than those that received two subcutaneous immunizations. However, the differences were not statistically significant. Nevertheless, this information confirms previous observations and highlights the importance of mucosal vaccine delivery to stimulate local immune responses. These results strongly suggest that the route of vaccine delivery is of paramount importance for development of an effective vaccine against pneumonic plague. The objective of this amendment is to reevaluate this vaccination approach using more animals and changes in the vaccine dose and the number of immunizations.

II. Objective

Assessment of protection against pneumonic plague following mucosal immunization of mice with the commercial *Yersinia pestis* (Greer Latoratories Inc.) formaldehyde-killed whole cell vaccine (KWC) formulated in liposomes.

III. Materials and Methods

Enzyme-linked immunosorbent assay (ELISA).

Antibodies specific for *Y. pestis* were determined by ELISA. Ninety-six well plates (Immulon 2; Dynatech Laboratories Inc., Alexandria, VA) were coated with *Y. pestis* antigens (100 ng/well) in a carbonate/bicarbonate buffer (pH 9.6). The plates were incubated overnight at 4 C and then washed 4 times in PBS-T. Four-fold dilutions of mouse sera were prepared in PBS-T (containing 0.5% gelatin) and dispensed in 100 l volumes. The plates were incubated for 2 h and washed. Affinity-purified horse anti-mouse IgG (H & L)-biotin conjugate (1/5,000; Vector Laboratories Inc., Toronto, Ont.) and anti-mouse IgA (1/5,000; Zymed, San Francisco, CA USA) were used as the detecting antibodies. After incubation for 2 h and four subsequent washes, a 1/10,000 dilution of streptavidin-alkaline phosphatase (Jackson Immunoresearch Labs.) in PBS-T (containing 0.5% gelatin) was added for 1 h. Di(Tris) p-nitrophenyl phosphate (PNPP, Sigma) was used as the chromogenic substrate. The absorbance was read after 10 minutes at 405 nm (BIO-RAD, Richmond, CA). Titres were expressed as the reciprocal of the dilution that intercepted the baseline (mean of negative control wells plus 3 S.D.).

Intranasal challenge with Y. pestis.

The 50% lethal dose (LD_{50}) was calculated according to the method described by Reed and Muench (Davis et al 1980). To evaluate immune protection against respiratory infection, immunized mice were intranasally challenged with *Y. pestis*. *Y. pestis* cultures were stored frozen on "Protect" bacterial preserver beads (TSC Ltd., Heywood UK). One to two beads were transferred to 10 ml of heart infusion broth and grown overnight at 28° C. One ml of this culture was transferred to 9 ml of heart infusion broth and grown overnight in a side-arm flask to an O. D. of 0.4. This O. D. corresponds to a concentration of $3x10^8$ colony-forming units (c.f.u.) Animals were anesthetized by metofane inhalation, and 25 l containing 6100 c.f.u. (10 LD_{50}) of *Y. pestis* strain GB (obtained from Chemical and Biological Defense Establishment, Porton Down, UK) was applied to the nares of each anesthetized mouse. Infected mice were closely monitored for signs of distress and symptoms of plague. Mice which show ruffled fur, hunched back, slow response to stimulus (e.g. being tourched and only moving slowly afterwards) were consider moribund and were killed

immediately. These end-points were in accordance with the guidelines of the Canadian Council on Animal Care. All surviving mice were killed on day 14 post-infection.

Statistical analyses

Data were entered into a database in the statistical analysis program Graph Pad InStat™ Version 3.00 (Graph Pad Software Inc. San Diego, CA). Differences in antibody titres were analyzed after logarithmic conversion by one-way ANOVA followed by Tukey post test; stimulation indices among vaccine groups were analyzed using the non-parametric Mann-Whitney U test and differences between survival curves were analyzed by log-rank test.

IV. Experimental Approach

Protection Studies:

Ability of 5μg of Y. pestis *vaccine to protect against intranasal challenge with live* Y. pestis.

Groups of mice will be immunized with a lower dose of antigen than previously used in the previous challenge experiment (5 μg vs 12 μg). We will assess the ability of this dose of *Y. pestis* vaccine to protect against the pneumonic plague. The following protocol will be used:

Group	Number	Route		Antigen (5 μg/dose) and formulation
		1st	2nd	
1	20 mice	s.c.	i.n.	PBS
2	"	s.c.	s.c.	*Y. pestis* vaccine alone
3	"	s.c.	i.n.	*Y. pestis* vaccine alone
4	"	s.c.	i.n.	*Y. pestis* vaccine-Liposome

Ability of a single immunization with 12μg of Y. pestis *vaccine to protect against intranasal challenge with live* Y. pestis.

Group	Number	Route	Antigen (12 μg/dose) and formulation
1	20 mice	s.c.	PBS
2	"	s.c.	*Y. pestis* vaccine alone
3	"	s.c.	*Y. pestis* vaccine-Liposome
4	"	i.n.	*Y. pestis* vaccine alone
5		i.n.	*Y. pestis* vaccine-Liposome

To assess the level of protection conferred by this immunization protocol, mice will be challenge by an intranasal exposure with 6100 cfu *Y. pestis* (10 MLD50) Animals will be kept in a level 3 facility at DRES, and will be observed daily.

V. Results

The capacity of KWC/Lipo to protect against pneumonic plague was evaluated with an intranasal *Y. pestis* challenge model. Mice were immunized by a s.c. injection of KWC, 21 days later they were boosted i.n. with KWC or KWC/Lipo. Control animals received PBS by the s.c. and i.n. routes. Two weeks after the second immunization all animals were challenge with an i.n. administration of 10 LD_{50} of *Y. pestis*. Two separate experiments were conducted. In the first trial, 12 animals/group were immunized with 12.5 μg of KWC as described above. More animals were protected from infection following i.n. immunization with KWC/Lipo (Fig. 7A, p=0.0008) than i.n. immunization with KWC in PBS (Fig. 7, p=0.018). In the second trial, the vaccine dose was lowered to 5 μg of KWC and the number of animals per group was increased to 20. The immunization protocol was the same as above. The results were similar to the first trial, in that more animals immunized i.n. with the KWC/Lipo were protected from aerosol infection than the group that received KWC in PBS (Fig. 7 B; p= 0.0001 vs 0.001 respectively). This protection was apparent as both an increased interval between challenge and death and reduced mortality.

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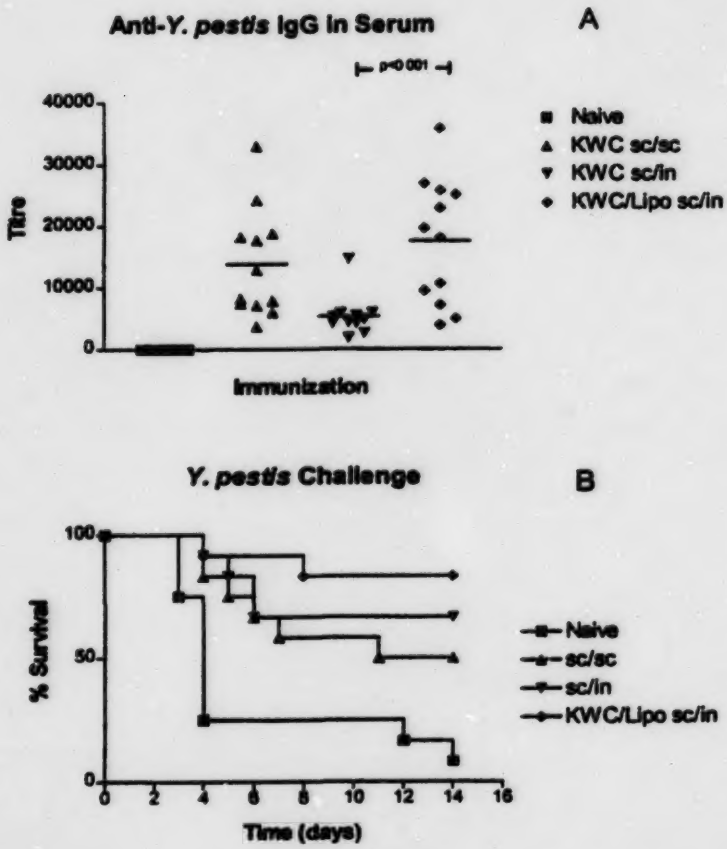

Figure 1. Systemic humoral immune responses (A) and protection against *Y. pestis* challenge (B).

Mice were immunized with 12.5 μg of *Y. pestis* vaccine by a combination of different routes. Animals received a primary immunization with KWC in PBS by the subcutaneous (s.c.) route and 21 days later were boosted s.c. (sc/sc) or intranasally (i.n.). Intranasally immunized mice received either KWC (sc/in) or KWC formulated in liposomes (KWC/Lipo sc/in). The titres of *Y. pestis*-specific IgG was determined in serum. Antibody titres are expressed as the mean titre ± SEM of 5 individual mice. Two weeks after the second immunization all animals were challenge with 10 LD_{50} of *Y. pestis*.

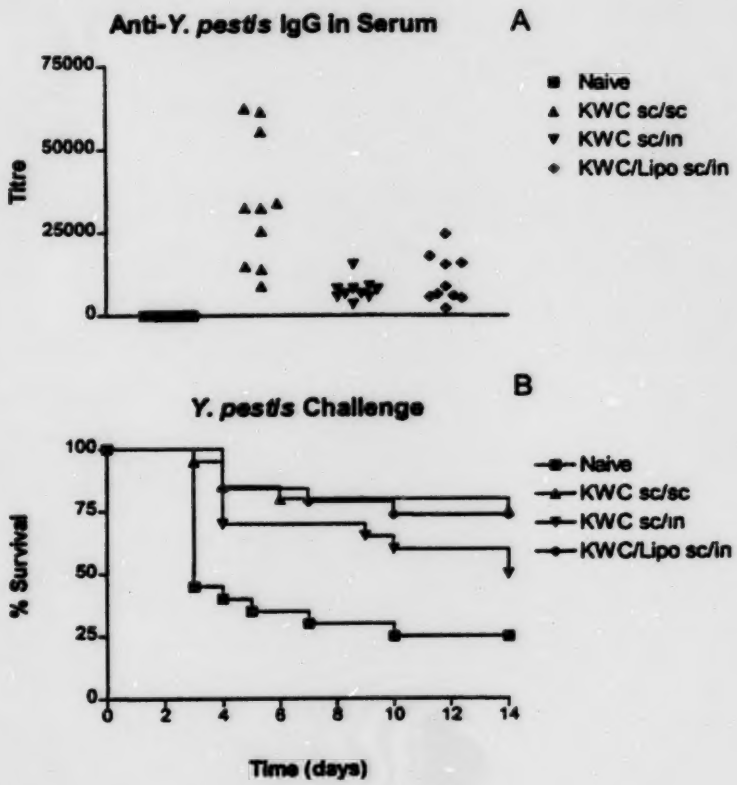

Figure 2. Systemic humoral immune responses (A) and protection against *Y. pestis* challenge (B).

Mice were immunized with 5 μg of *Y. pestis* vaccine by a combination of different routes. Animals received a primary immunization with KWC in PBS by the subcutaneous (s.c.) route and 21 days later were boosted s.c. (sc/sc) or intranasally (i.n.). Intranasally immunized mice received either KWC (sc/in) or KWC formulated in liposomes (KWC/Lipo sc/in). The titres of *Y. pestis*-specific IgG was determined in serum. Antibody titres are expressed as the mean titre ± SEM of 5 individual mice. Two weeks after the second immunization all animals were challenge with 10 LD_{50} of *Y. pestis*.

VII. Discussion

The primary aim of this study was to determined if the route of delivery and formulation could alter the induction of systemic and mucosal immune responses to a commercial *Yersinia pestis* vaccine. Our results clearly demonstrated that a single i.n. immunization with liposome-formulated *Y. pestis* vaccine, induced a primary mucosal immune response in the respiratory tract. Those animals that were i.n. immunized developed antigen-specific antibody-secreting cells in the lungs, indicating that local production of specific antibody was stimulated following i.n. administration. Similarly, effective enhancement of mucosal immune responses was also observed with two i.n. immunizations using lower doses of antigen formulated in liposomes. Thus, liposome formulation of a commercial KWC vaccine provided a very efficient delivery system for induction of mucosal immunity. No detectable antigen-specific respiratory IgA responses developed following systemic vaccination. The information presented here confirms previous studies (reviewed in Kiyono et al 1996) and highlights the importance of mucosal vaccine delivery to stimulate local immune responses.

Another important finding in this study is the observation that formulation of the *Y. pestis* vaccine in liposomes significantly enhanced its immunogenicity following i.n. delivery. Enhanced immunogenicity may be crucial for reducing the dose needed to induce protective immunity and decreasing the adverse reactions frequently associated with the current *Y. pestis* vaccine (Perry et al 1997). Recent studies have shown that intranasal administration of microsphere co-encapsulated *Y. pestis* subunit vaccines enhances resistance against pneumonic plague in the mouse (Eyles et al 1998). However, full protection against the disease was only achieved when free *Y. pestis* subunits were administrated together with cholera toxin B subunit (Eyles et al 1998). In addition, i.n. immunization with liposomes, surface-labeled with F1 antigen, conferred partial protection against a s.c. challenge with *Y. pestis* (Reddin et al 1998) but this study did not assess protection against pneumonic plague.

The ability of liposomes to enhance immune responses to vaccine antigens has been attributed primarily to an increased antigen uptake by antigen presenting cells and consequently increased antigen presentation to T cells (reviewed in Gregoriadis 1990). DDA is a lipid component of the liposome formulation used in this experiments. Since DDA has been shown to have adjuvant properties (Gordon et al 1980; Prager and Gordon 1978), it is possible that the presence of DDA in the liposome formulation may have contributed to the enhancement of the immune responses observed in these

studies. However, previous studies have shown that immune responses induced by subcutaneous immunization with liposome formulations of a viral subunit vaccine containing DDA did not differ from the responses induced by liposome formulations without DDA (Baca-Estrada et al 1997).

We chose to assess protection using a s.c. priming with KWC in PBS followed by an i.n. boost. This protocol was selected since many people have been immunized intramuscularly with the current *Y. pestis* vaccine. Therefore, it was considered important to evaluate immune protection against pneumonic plague when systemic vaccination with KWC alone was combined with an i.n. boost. More animals were protected after intranasal immunization with KWC/Lipo vaccine than i.n. immunization with KWC in PBS in two separate experiments. However, there was no significant difference between the two immunized groups.

The cellular immune response elicited by the KWC vaccine was characterized by the induction of a strong IFN-γ secretion with no IL-4 secreting cells detected. The implications of these findings for the use of *Y. pestis* vaccines formulated in liposomes are important, since enhanced Th1 responses should contribute to protection against an intracellular pathogen such as *Y. pestis* (Nakajima and Brubaker 1993). Interestingly, formulation of KWC with liposomes did not change the pattern of the Th response induced by KWC. The preferential induction of IFN-γ may have been meditated by potent immune stimulants found in the KWC vaccine, including LPS and bacterial DNA.

In conclusion, these results strongly suggest that the route of vaccine delivery and vaccine formulation may be of paramount importance when developing an effective vaccine against pneumonic plague.

VI. References

Baca-Estrada ME, Foldvari M, Snider M, van Drunen Littel-van den Hurk S and Babiuk, LA. Effect of IL-4 and IL-12 on the induction of immune response to bovine herpesvirus type-1 glycoprotein D. Vaccine 1997;15:1753-60.

Davis B, Dulbecco R, Eisen HN and Ginsberg HS. 1980. Microbiology. Harper & Row Publishers Inc., Hagerstown, p. 883.

Eyles JE, Sharp GJE, Williamson ED, Spiers ID and Alpar HO. Intra nasal administration of poly-lactic acid microsphere co-encapsulated *Y. pestis* subunits confers protection from pneumonic plage in the mouse. Vaccine 1998;16:698-707.

Gordon WC, Prager MD and Carroll MC. The enhancement of humoral and cellular immune responses by dimethyldioctadecylammonium bromide. Cell Immunol 1980;49:329-40.

Kiyono H, Ogra PL and McGhee, JR. 1996. Mucosal Vaccines. Academic Press, San Diego, CA.

Nakajima R and Brubaker RR. Association between virulence of *Yersinia pestis* and suppression of gamma interferon and tumor necrosis factor alpha. Infect. Immun. 1993;61:23-31.

Perry RD and Fetherston, JD. *Y. pestis*-etiologic agent of plague. Clin. Microbiol. Rev. 1997;10:35-66.

Prager MD and Gordon WC. Enhanced response to chemoimmunotherapy and immunoprophylaxis with the use of tumor-associated antigens with a lipophilic agent. Cancer Res 1978;38:2052-7.

Reddin KM, Easterbrook TJ, Eley SM, Russell P, Mobsby VA, Jones DH, Farrar GH, Williamson ED and Robinson, A. Comparison of the immunological and protective responses elicited by microencapsulated formulations of the F1 antigen from Yersinia pestis. Vaccine 1998;16:761-7.

Research Report – Appendix A to Annex A

Evaluation of Mucosal Immune Responses to *Yersinia pestis* Vaccine

I. Introduction

Current immunization protocols using intramuscular (IM) injections with *Yersinia pestis* vaccine induce a strong systemic antibody and cellular immune responses that can be measured in the serum and the peripheral lymphoid tissues. These immune responses are effective in protecting against the systemic form of the disease transmitted by the bites of infected fleas (bubonic plague). However, vaccinated individuals are susceptible to the respiratory form of *Y. pestis* infection, which is transmitted through airborne droplets from infected individuals (pneumonic plague). Thus, IM vaccination induces systemic immunity but this does not prevent *Y. pestis* invasion of the mucosal surfaces in the lung.

Important advances have been made in understanding the mechanisms responsible for the induction of mucosal immune responses. It is well recognized that i.m. injection of vaccine antigens usually fails to induce mucosal immunity. Investigations in numerous laboratories have proven that antigen delivery through the inductive sites of the gastrointestinal or respiratory tracts is necessary for the induction of mucosal immunity. Thus, the mucosal immune system often functions separately from the systemic immune system.

II. Objectives

The overall objective of the contract "Development of a Mucosal Vaccine Vector for Yersinia pestis" (W7702-5-R527/01-XSG) is to develop viral and bacterial vectors for delivery of *Y. pestis* antigens to induce mucosal immunity. The present contract amendment was initiated with the specific aim to establish the necessary immunological assays to evaluate humoral (antibody) and cellular (T cell) immune responses to *Y. pestis* in mice and to further assess whether the route of delivery and formulation of *Yersinia pestis* vaccine could alter the induction of systemic and mucosal immune responses.

The work carried out in this project established the necessary immunological techniques and expertise required for the in vivo evaluation of the recombinant vector vaccines currently being developed at VIDO. Furthermore, these techniques were used to clearly establish that the mucosal vaccine delivery is necessary to induce local immune responses in the respiratory tract. This information is vital for the development of a vaccine that can prevent pneumonic plague.

III. Materials and Methods

To address the objectives of this proposal the following assays were developed:

a) To evaluate the induction of humoral responses to *Y. pestis*.

ELISA to measure (IgG, IgA, IgM, IgG1, IgG2a, IgG2b and IgG3).

ELISPOT to detect (IgG and IgA antibody secreting cells in lungs and spleen).

b) To evaluate the induction of cellular immune responses to *Y. pestis*.

T-cell proliferative responses.

ELISPOT to detect (IFN-γ and IL-4 secreting cells in lungs and spleen).

c) Development of multilamellar (MLV) liposomes containing *Y. pestis* vaccine antigens (Plague Vaccine USP, Greer Laboratories, Inc. Lot # 1128X1).

IV. Results

Experiment # 1.

Assessment of dose response to* Y. pestis *vaccine incorporated into MLV liposomes.

The efficiency of incorporation of the *Y. pestis* bacterin into the vesicles was determined by using a protein assay. The amount of incorporated antigen was calculated by the proportion of protein found in the pellet and supernatant following centrifugation of liposome/bacterin preparations (Fig 1A).

To assess the potency and immunogenicity of the vaccine-liposome formulation we immunized mice subcutaneously with three different formulations. These contained 3.2, 32 and 150 μg of protein and had a lipid:protein ratio of approximately 1000, 100 and 20, respectively. The liposome/bacterin preparations were washed and a protein assay was performed with the supernatant. Animals immunized with these formulations developed a dose dependent *Y. pestis*-specific serum antibody response (Fig. 1B). Although the percent incorporation, determined by the protein assay, correlated with the ability of these formulations to induce an immune response, subsequent experiments were not consistent. Therefore, our ability to estimate the amount of encapsulated bacterin was not dependable, and liposome/bacterin preparations used in further experiments were not washed.

Antigen-specific proliferative responses were also established during these early experiments (Fig1C). Strong proliferative responses were induced in naïve (PBS- immunized) mice at the protein concentrations used in this experiment.

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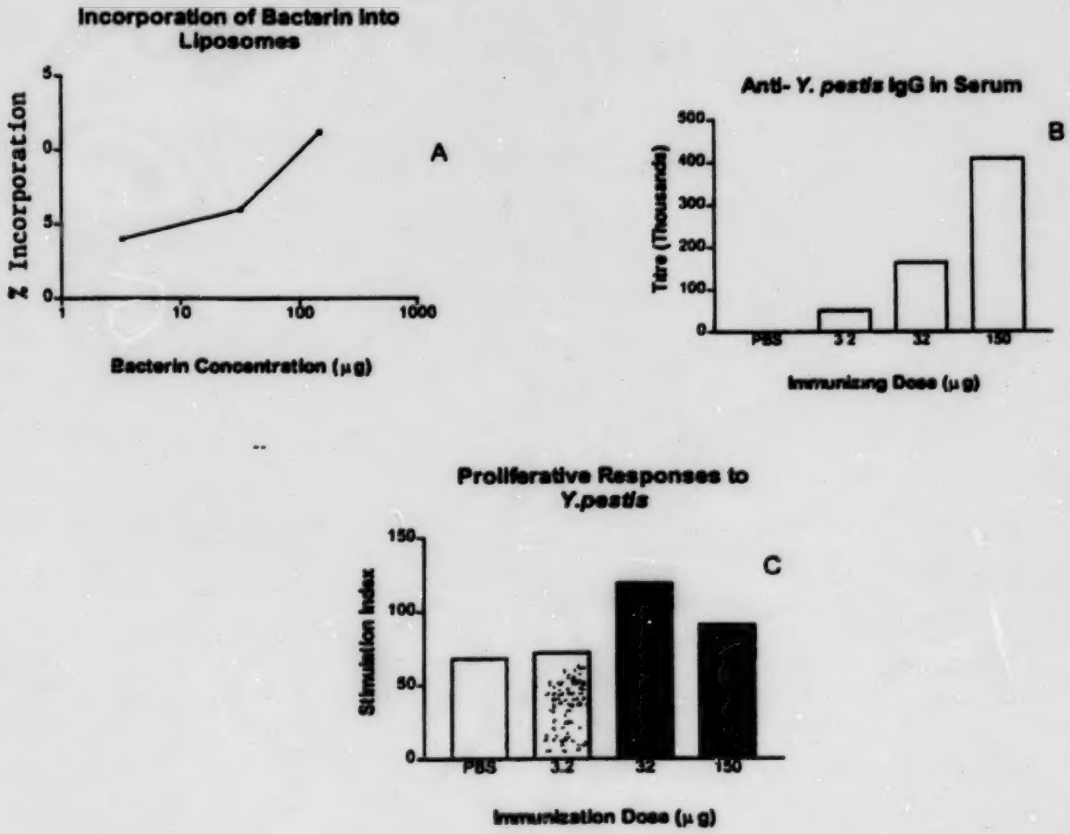


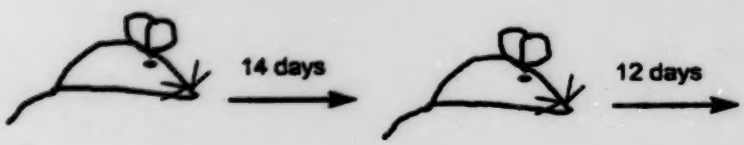
Figure 1. Incorporation efficiency and immune response to different doses of liposome/bacterin formulations.

(A) Liposome incorporation efficiency of increasing amounts of bacterin was determined by a protein assay. % Incorporation = Total protein - protein in supernatant/total protein x 100. (B) The titre of *Y. pestis*-specific IgG in serum of mice immunized with increasing amounts of bacterin incorporated in liposomes. (C) Mouse splenocytes proliferative responses following a single subcutaneous (SC) immunization with *Y. pestis* antigens incorporated into liposomes. Stimulation index = cpm in the presence of antigen/cpm in media alone. The bacterin induced a strong non-specific proliferation of splenocytes in naïve (PBS) mice. Results for B and C represent the mean of two mice per group.

Experiment # 2.

Assessment of optimal route of delivery to induce Y. pestis-specific immune responses in the respiratory tract.

The following experimental approach was used to evaluate the optimal route of vaccine delivery to induce mucosal and systemic immune responses against *Y. pestis* vaccine antigens. Mice were immunized with *Y. pestis* vaccine, formulated in liposomes, by either the intranasal (IN) or a combination of subcutaneous (SC) and IN routes.



1 PBS IN	1 PBS IN	Cont	Mucosal immune response
2 Bact/Lipo SC (50 ug)	2 Bact/Lipo IN (12 5 ug)	SC/IN	Lung and nasal washes
3 Bact/Lipo IN (12.5 ug)	3 Bact/Lipo IN (12 5 ug)	IN/IN	Lung cells
			Systemic immune response
			Serum
			Spleen cells

The results of these experiment indicated that a primary SC followed by an IN boost induced significantly higher levels of humoral (Fig 2) and cellular (Fig 3) responses in the respiratory tract than two IN immunizations. Cellular immune responses were assayed by the production of specific cytokines following an in vitro stimulation with *Y. pestis* bacterin. The cytokine (CK) secreting cells were detected with an ELISPOT assay specific for either IL-4 or IFN-γ (Fig 3). It is important to note that the amount of antigen in the primary IN immunization was one quarter of the SC dose.

Mucosal Immune Responses

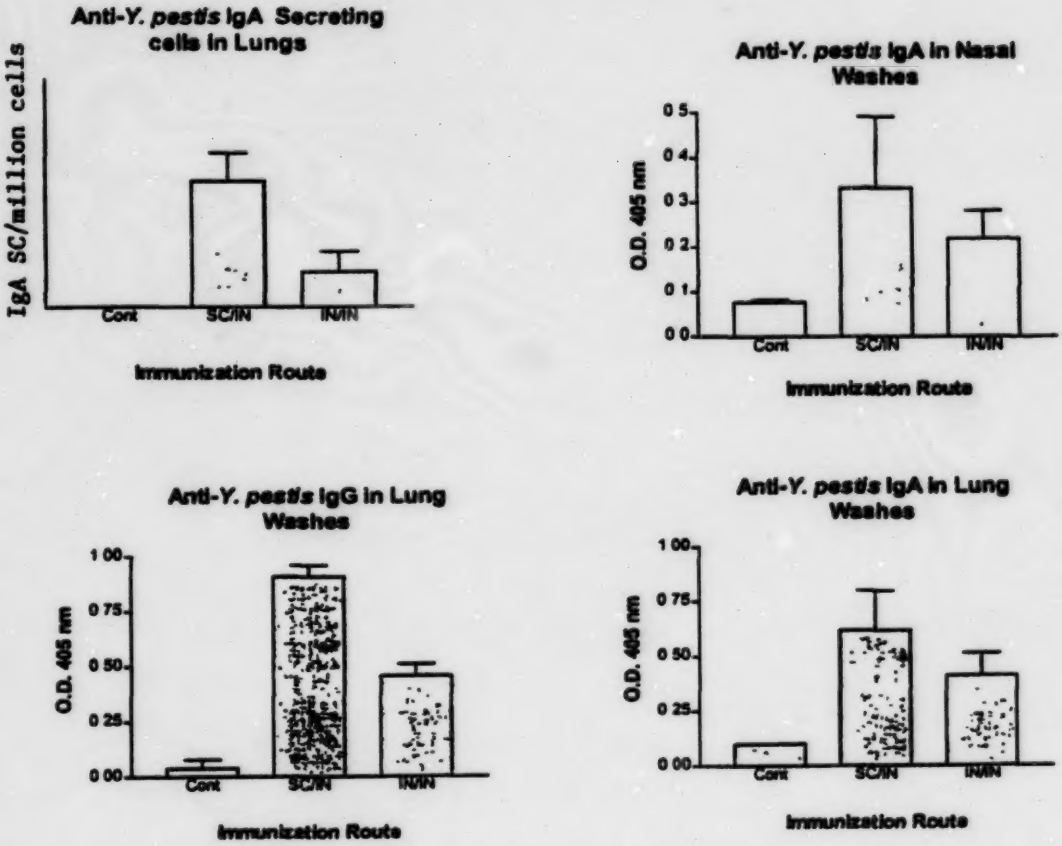


Figure 2. Effect of immunization route on the induction of humoral immune responses in the mucosa.

(A) The frequency of *Y. pestis*-specific IgA-secreting cells in the lungs of immunized mice; (B) The level of anti-*Y. pestis* IgA in nasal washes of immunized mice. Nasal washes were obtained by injecting 0.3 ml of PBS into the trachea towards the nose and collecting the fluid in a microfuge tube as it exited the nares; (C) Anti-*Y. pestis* IgG and (D) Anti-*Y. pestis* IgA in lung washes of immunized mice. Lung washes were obtained by gently injecting 0.5 ml PBS into the trachea, to expand the lungs. The lavage was repeated by aspirating the fluid back into the syringe and reinjecting once more into the lungs before final withdrawal. The results represent the mean ± sem of the optical density (O.D. 405 nm) measured in samples diluted 1:10.

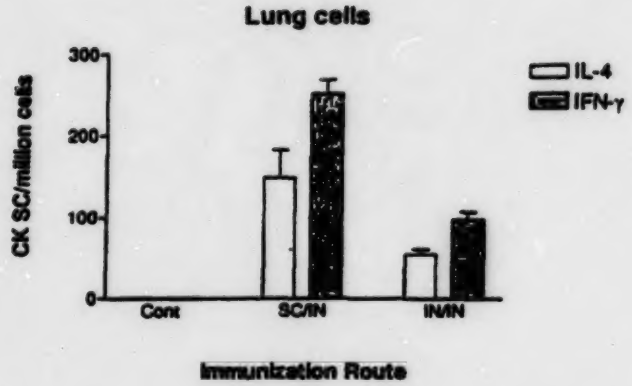


Figure 3. Effect of route of immunization in the induction of antigen-specific cellular immune responses in the respiratory tract.

The cellular immune response to *Y. pestis*/liposome vaccine is characterized by the induction of significantly higher levels of IFN-γ-secreting cells than IL-4-secreting cells in the lungs of immunized mice.

Systemic Immune Responses

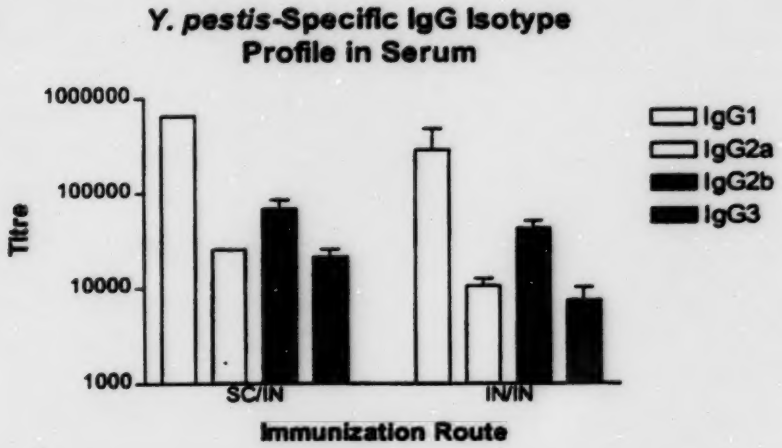


Figure 4. ***Y. pestis*-specific IgG isotype profile in serum of immunized mice.**

The strong antigen-specific IgG response was characterized by the induction of all IgG isotypes with significantly higher levels of IgG1 ($p<0.05$) compared to the other isotypes.

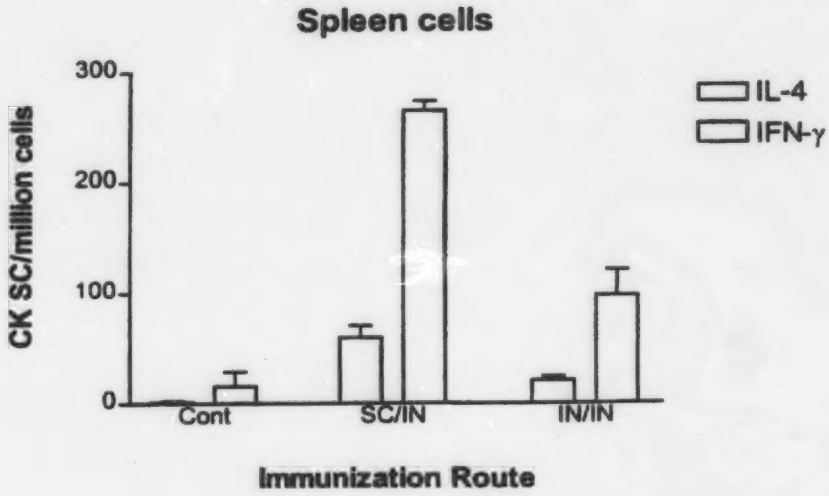


Figure 5. Effect of route of immunization in the induction of antigen-specific cellular immune responses in the spleen of immunized mice.

The cellular immune response to *Y. pestis*/liposome vaccine is characterized by the induction of significantly higher levels of IFN-γ-secreting cells than IL-4-secreting cells regardless of route of immunization.

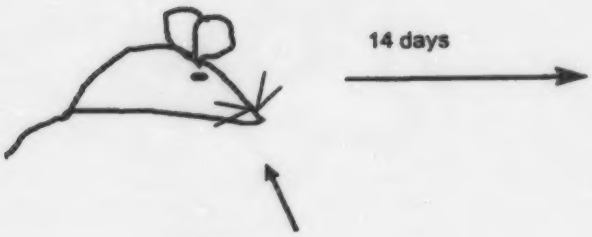
Summary:

1. A primary immunization either SC or IN, followed by an IN boost induces both mucosal and systemic immune responses to *Y. pestis* antigens.
2. Mucosal immune responses included a high frequency of IgA secreting cells in the lung, high levels of specific-antibodies in the mucosal secretions and a high frequency of IFN-γ secreting cells in the lung.
3. Systemic immune responses included the induction of antigen-specific IgG antibodies of all isotypes and predominantly IFN-γ producing cells in the spleen.

4. ***Experiment # 3.***

Effect of liposomal encapsulation of* Y. pestis *vaccine on the induction of mucosal and systemic immune responses following intranasal immunization.

To assess whether encapsulation of *Y. pestis* vaccine into MLV liposomes had an effect on the levels and the type of immune response elicited, we immunized mice IN with 12.5 μg of *Y. pestis* vaccine or 12.5 μg of bacterin encapsulated into MLV liposomes.



Mucosal immune response:
Lung and nasal washes
Lung cells
Systemic immune response:
Serum
Spleen cells

1. PBS
2. Bact-12.5 ug
3. Bact-12.5 ug/Liposomes (Bact/Lipo)

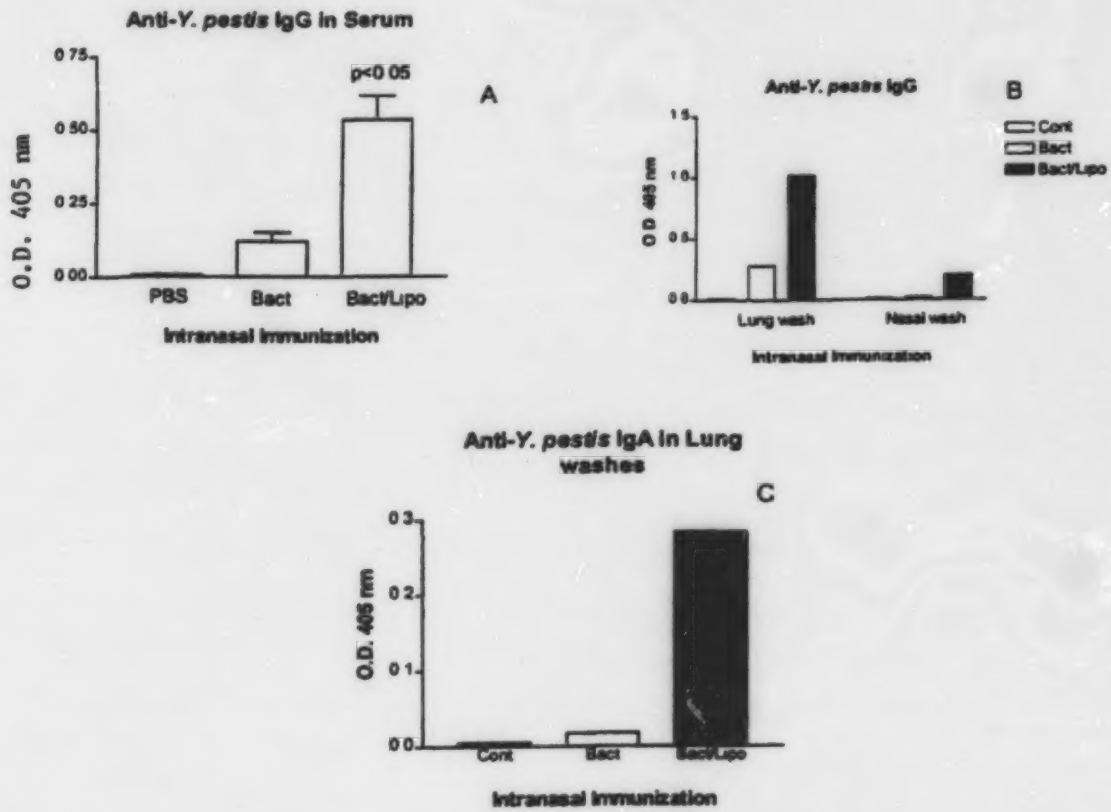


Figure 6. *Y. pestis*-specific antibody response measured by ELISA, following intranasal immunization with vaccine alone (Bact) or vaccine formulated in MLV liposomes (Bact/Lipo).

A significant increase ($p<0.05$) in serum antibodies were observed in mice immunized with Bact/Lipo compared to those that received Bact alone (A). The Bact/Lipo group also displayed an increase in the levels of specific IgG in lung and nasal washes (B) and increased amounts of specific IgA in lung washes with three out of five mice having detectable levels of IgA. Antibody levels are expressed as the optical density (O. D. 405 nm) measured in diluted samples. Individual serum samples (5 mice/group) were diluted 1:10240 and expressed as the mean ± sem. Lung and nasal washes were pooled and diluted 1:10.

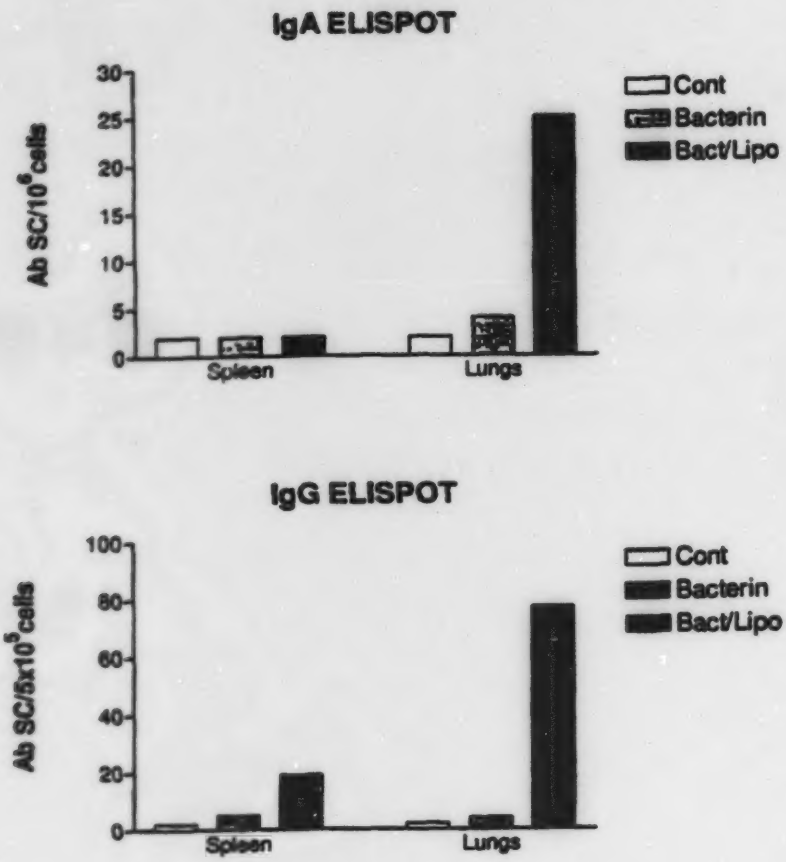


Figure 7. *Y. pestis*-specific antibody response measured by ELISPOT, following intranasal immunization with vaccine alone (Bact) or vaccine formulated in MLV liposomes (Bact/Lipo).

We used an ELISPOT to assess the number of anti-*Y. pestis* IgG and IgA secreting cells in the spleen and lungs of immunized mice. An increased number of antibody secreting cells (Ab SC) were observed in mice that were immunized with the Bact/Lipo vaccine.

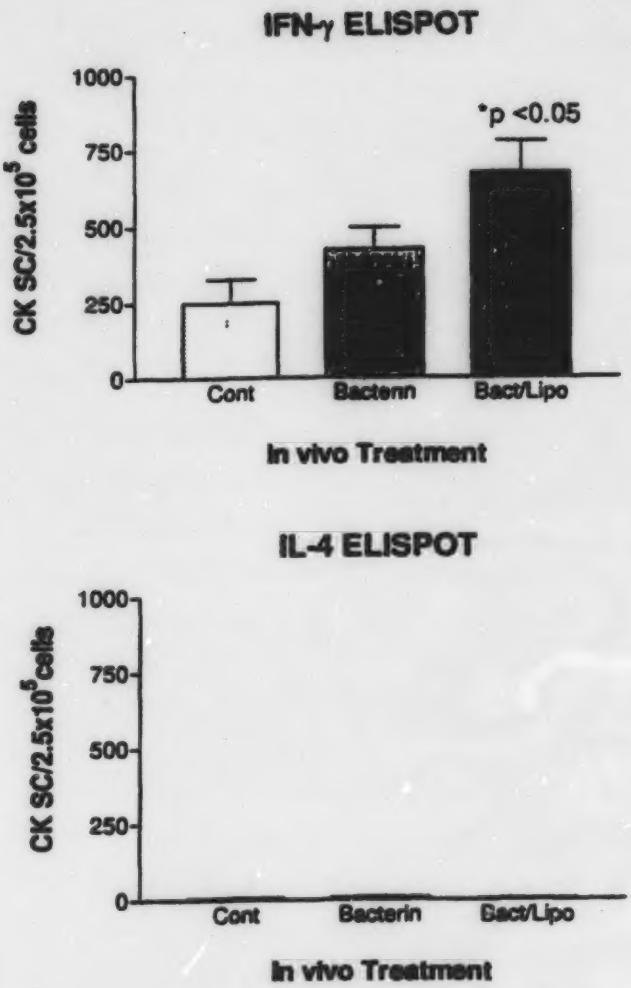


Figure 8. Effect of liposomal encapsulation of *Y. pestis* vaccine in the induction of antigen-specific cellular immune responses in the spleen of immunized mice.

The cellular immune response to *Y. pestis* vaccine, both alone or encapsulated into liposomes, is characterized by the induction of significantly higher levels of IFN-γ-secreting cells than IL-4-secreting cells. Furthermore, mice immunized with Bact/Lipo formulation had significantly ($p<0.05$) more IFN-γ producing cells than those immunized with Bact alone. In this experiment we observed high levels of non-specific stimulation of IFN-γ producing cells in the control group.

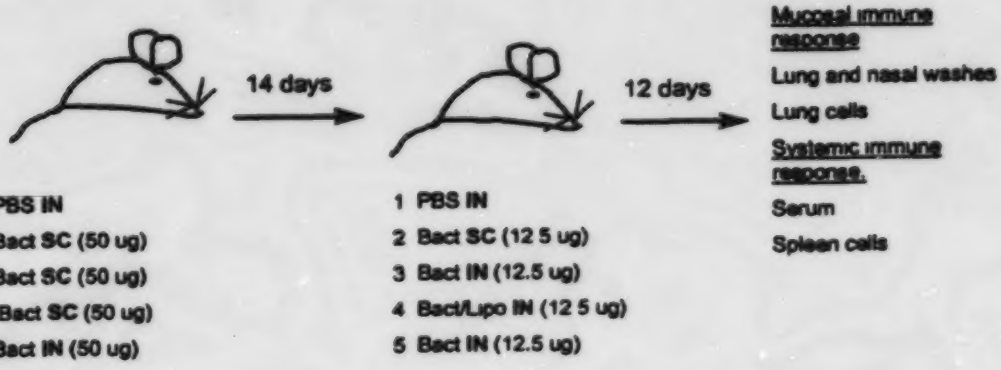
Summary:

1. A single IN immunization with bacterin alone induced low levels of cellular and humoral immune responses.
2. A single IN immunization with bacterin incorporated into liposomes induced strong cellular and humoral immune responses.
3. The liposome encapsulated bacterin induced both mucosal and systemic immune responses.
4. The ELISPOT results confirmed the ELISA data and further indicated that the respiratory tract is a major source of specific antibody following IN immunization.

Experiment # 4.

Evaluation of the optimal route of delivery and vaccine formulation for the induction of mucosal immunity to* Y. pestis *vaccine.

The final experiment was designed to evaluate the influence of both route of delivery and formulation of the vaccine on the induction of mucosal immunity. Since many individuals have been already immunized with the current *Y. pestis* vaccine, it was important to evaluate the impact of using the current vaccine protocol with an additional intranasal boost, on the induction of mucosal immunity in the respiratory tract. Therefore, mice were immunized with the same amount of bacterin (12.5 µg) by the SC or IN route, and then boosted as described in the following layout:



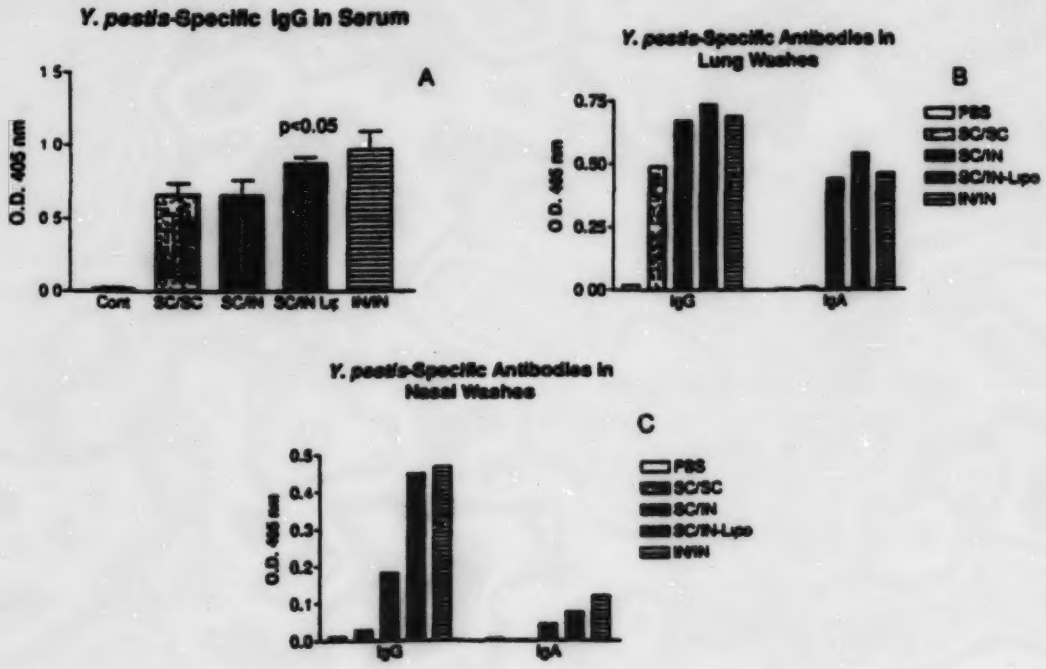


Figure 9. Systemic and mucosal humoral immune responses in mice immunized with *Y. pestis* vaccine by different routes of delivery.

All immunized groups developed high levels of specific IgG antibodies in serum, however, those animals immunized SC and boosted with Bact/Lipo formulation had significantly ($p<0.05$) higher antibody levels than those immunized SC with bacterin alone (A). Assessment of specific IgG and IgA antibody in the lung washes (B) and nasal washes (C) of immunized mice indicated that induction of IgA responses in respiratory tract secretions was dependent on the mucosal delivery of the vaccine. Antibody levels are expressed as the optical density (O. D. 405 nm) measured in diluted samples. Individual serum samples (5 mice/group) were diluted 1:10240 and expressed as the mean ± sem. Lung and nasal washes were pooled and diluted 1:10.

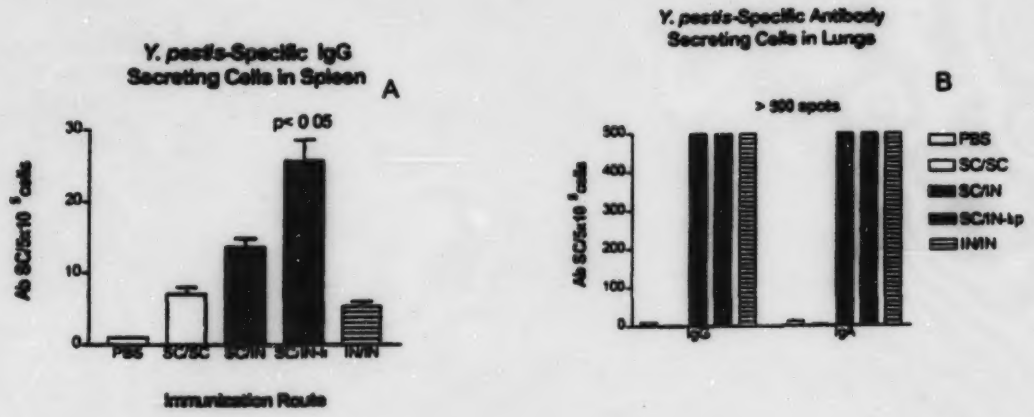


Figure 10. Systemic and mucosal humoral immune responses measured by ELISPOT assay in mice immunized with *Y. pestis* vaccine.

All immunized groups showed high numbers of specific IgG antibody secreting cells in the spleen. Interestingly, the number of specific antibody secreting cells in the spleen revealed a significant ($p<0.05$) increase in of mice that were SC primed and boosted IN with Bact/Lipo vaccine (A). Assessment of specific IgG and IgA antibody secreting cells in the lungs of immunized mice indicated that the induction of IgA secreting cells responses in the respiratory tract was dependent on the mucosal delivery of the vaccine. Unfortunately the number of antibody secreting cells in the lungs of these animals was very high and we were unable to evaluate differences among the groups at the dilution of cells used in the assay.

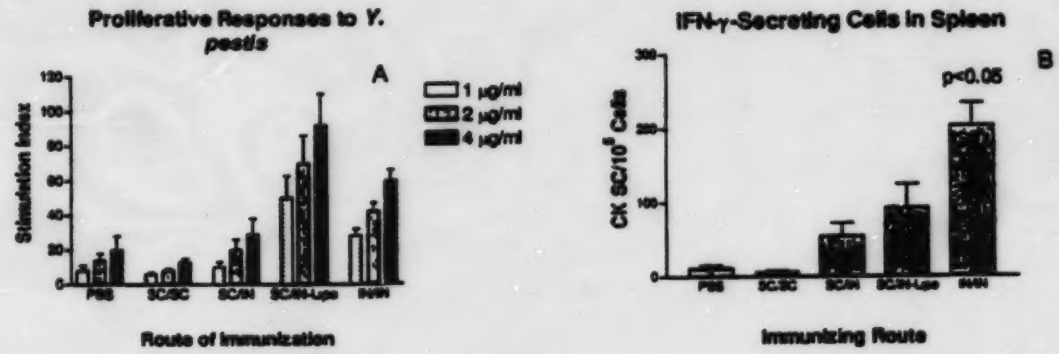


Figure 11. The effect of vaccine delivery and formulation on cellular immune responses to *Y. pestis*.

Proliferative responses to *Y. pestis* were dose dependent to the antigen used in the assay and were significantly ($p<0.05$) higher proliferative responses were observed in the groups SC/IN-Lipo or IN/IN (A). The *Y. pestis*-specific cellular responses were characterized by secretion of high levels of IFN-γ (B). No IL-4 secretion was observed in this experiment (data not shown).

Summary:

1. Intranasal delivery of *Y. pestis* antigens is essential for induction of specific IgA in the mucosal secretions of the respiratory tract and the localization of specific IgG and IgA secreting cells in the lungs.
2. Intranasal delivery induced a strong cellular immune response. This response was characterized by T-cell proliferative responses and IFN-γ secretion.
3. Incorporation of bacterin into liposomes significantly enhanced the immune response to *Y. pestis* antigens.

VI. SUMMARY AND CONCLUSIONS

The aim of this proposal was to assess whether the route of delivery and formulation of *Yersinia pestis* vaccine could alter the induction of systemic and mucosal immune responses. Our results demonstrated that induction of mucosal immunity in the respiratory tract is efficiently induced by intranasal delivery of the vaccine. In contrast, no specific respiratory responses could be induced following systemic vaccination (SC). This information confirms previous observations and highlights the importance of mucosal vaccine delivery to stimulate local immune responses.

The results obtained in our preliminary experiment (#2) suggested that systemic priming (SC) followed by an intranasal boost induced significantly higher mucosal immunity than two intranasal vaccinations (Fig 2, 3 and 5). These results differ from those obtained in our last experiment (#4) where two intranasal immunizations were as efficient or better than SC and intranasal deliveries. These conflicting results may be explained by the fact that in the initial experiment the dose given for SC priming was 50 μg and the dose for intranasal priming was 12.5 μg. For the last experiment (#4) we administered (50 μg) for the IN and SC priming immunization.

Another important finding in this study is the observation that formulation of the *Y. pestis* vaccine in liposomes significantly enhanced its immunogenicity following IN delivery. Enhancement of immunogenicity may be crucial in reducing the dose needed to induce protective immunity and decrease the adverse reactions frequently associated with the current vaccine.

IFN-γ is a crucial cytokine involved in the protection against intracellular pathogens such as *Y. pestis*. We have demonstrated that the cellular immune response elicited by the vaccine is characterized by the induction of a strong IFN-γ secretion. This response is antigen-specific and may contribute to the protection against *Y. pestis* infection.

In conclusion, the present vaccine for *Y. pestis* induces systemic immunity but no mucosal responses when delivered IM or SC. However, we found that the same vaccine antigen, when delivered IN, induced strong mucosal immunity in the respiratory tract. These results strongly suggest that the route

of vaccine delivery is of paramount importance for development of an effective vaccine against pneumonic plague. A final conclusion on this matter must wait until experiments are concluded to prove that the induction of mucosal immunity can protect mice against an aerosol challenge with *Y. pestis* (pneumonic plague model).

Research Report – Appendix B to Annex A

Development of Mucosal Vaccines for *Yersinia pestis*

I. Objectives

The objectives for Appendix B to Annex A were divided into two sections as follows:

Part I. Regulatory Issues for *Y. pestis* Vaccines

1. Identification of the regulatory steps required to gain approval for use of the modified vaccine i humans.
2. Development of a plan which details the necessary experiments required in order to obtai regulatory approval.
3. Prepare a report based on the results obtained from objectives (1) and (2) above.

Part II. Protective capacity of *Y. pestis* antigens

4. Purify recombinant subunit vaccines and establish immunological assays in preparation fo challenge studies.
5. Establish a *Y. pestis* aerosol challenge model in mice.
6. Conduct protection studies using the model established in (5).
7. Prepare a final report based on objectives (4), (5) and (6) above.

Research Report – Appendix B to Annex A, Part I

***Regulatory issues for *Y. pestis* Vaccines**

*A report addressing the regulatory issues facing licensing of plague vaccines plus experiments which would need to be carried out was prepared by CANTOX, Inc. and is appended on the following pages.

CANTOX INC.

Consultants in Toxicology
Health and Environmental Sciences

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PRIVILEGED AND CONFIDENTIAL

May 4, 1998

Dr. Lorne Babiuk
Director
Veterinary Infectious Disease Organization
120 Veterinary Road
Saskatoon, Saskatchewan
S7N 5E3

RE: REGULATORY AND SCIENTIFIC REQUIREMENTS FOR THE REGISTRATION OF A MODIFIED PLAGUE VACCINE

Dear Dr. Babiuk:

CanTox has now completed its assessment of the information and data which would be anticipated to be necessary to support the registration in Canada for use in humans of a modified plague vaccine. As requested, the purpose of our review was (1) to identify the regulatory steps required to gain approval of this product and (2) to propose a plan which details the necessary experiments and studies to support registration. The product under development is a modification of Plague Vaccine U.S.P., manufactured by Greer Laboratories Inc., reformulated in a lipososomal delivery system, for administration by the intranasal route for induction of mucosal immunity against pneumonic forms of plague (*Yersinia pestis* infection). It is currently proposed that the intranasal administration of the modified vaccine (or possibly the approved fromulation) would form adjunct therapy to the current regimen of intramuscular injection of Plague Vaccine U.S.P., which is licensed in Canada, the United States, and elsewhere, using intramuscular injection only. As such, the modified vaccine represents a situation where both the formulation (novel) and the route of administration are significantly different from that of the licensed product.

While we have focussed on registration in Canada, it should be noted that the points that will be raised and discussed, as well as the scientific and regulatory needs that will reviewed will apply to other jurisdictions, such as the United States. The following outlines the results of our findings and is divided into sections for the sake of clarity.

Approval of Vaccines in Canada

The review and approval of vaccines in Canada fall under the *Food and Drugs Act* and *Regulations*, and are the responsibility of the Vaccines Division, Bureau of Biologics and Radiopharmaceuticals (BBR) of the Health Protection Branch, Health Canada. The current Chief of this Division is Dr. Laszlo Palkonyay, while the individual responsible for bacterial products is Dr. Jerry Calver.

In general, the following outlines the steps involved in the development and approval of a novel vaccine (which can take up to 10 years). Please note that certain steps will not be necessary with the proposed modified vaccine, since the starting material, Plague Vaccine U.S.P., is manufactured as a sterile product under Good Manufacturing Practices (GMP) and has been licensed for over 30 years:

1. Exploratory (basic) research

2. Developmental research
   - isolation and definition of disease-producing agent
   - methodology of propagating organism/expressing antigen
   - markers for protective immunity identified
   - animal models established

3. Preclinical testing
   - safety and tolerance in animals
   - characterization of vaccine (*e.g.*, absence of infectious agents, purity, potency, stability)

4. Manufacturing under GMP regulations
   - includes final formulation
   - all lots of vaccine intended for investigational testing in humans must be prepared under GMP
   - issues include (preservatives and potency, adjuvants, inactive compounds, stability/expiration dating)
   - will need to make reference to the proprietary data of Greer Laboratories Inc.

5. Interaction with BBR regarding clinical trials
   - preparation and filing of pre-Investigational New Drug (pre-IND) packages
   - Pre-IND meetings with BBR staff
   - Preparation and filing of INDs (prepared in the required format as per applicable guidelines and regulations)

6. Conduct of clinical trials
   - Must be carried out under Good Clinical Practices (GCP) regulations
   - Phases:
     - I tolerability and immunogenicity established in small numbers of healthy adults
     - II definitive establishment of immunogenicity, safety, tolerability, dosage, dosage regimen, and final formulation
     - III establishing the protective efficacy of the vaccine in a blinded, placebo-controlled manner (not applicable in the case of a plague vaccine)

7. Interaction with BBR regarding approval
   - pre-New Drug Submission (pre-NDS) meetings with BBR staff
   - preparation and filing of NDS (prepared in the required format as per applicable guidelines and regulations)
   - responding to questions

8. Post approval
   - Lot release
   - Post marketing surveillance and reporting

While Plague Vaccine U.S.P. has a long history of use and is licensed in Canada, the fact that the intranasal route of administration proposed for the modified vaccine differs from that which is currently approved and the fact that the formulation will be substantially different from that which is approved, means that the new vaccine will be considered a "new drug" and an NDS will be required. In contrast, utilizing a new route of administration or a new immunization protocol (*i.e.*, administration of the currently approved vaccine formulation by the intranasal route in addition to the intramuscular injections) for an approved vaccine formulation for the same indication could be accomplished through a Supplemental NDS (SNDS), and all that would be required would be demonstrating, through the use of surrogate data, that the same level of

systemic immunity was conferred (*i.e.*, for treating the same disease). It should be noted that if such a change was implemented for a new indication, clinical data supporting the effectiveness of the product for the new use is required (see below). In the case of plague vaccine, protection against the pneumonic form of the disease would constitute a "different disease" requiring supporting data.

Currently, HPB has a cost recovery program in place, so the fees that Sponsors must pay in order to have a submission reviewed would range from $68,200 to $117,000 depending on their interpretation of the categories of data that require review, yet it is possible to apply for a reduction of fees.

There are a number of guidance documents published by Health Canada governing the content and format of IND and NDS submissions, including electronic submission templates, that CanTox can make available should VIDO not be in possession of these documents.

Novel Adjuvants

From discussions with Dr. Philip Griebel of the Veterinary Infectious Disease Organization (VIDO), it is our understanding that the new vaccine would be produced by a proprietary process in which Plague Vaccine U.S.P., purchased from the manufacturer and with no intermediate steps, would be used as a starting material for production of a liposomal formulation. Based upon this, all indication is that humans would not receive higher doses of the antigens (and other components of Plague Vaccine U.S.P., such as formaldehyde and phenol) than delivered by the approved vaccine, albeit by a different route of administration. While this information and the long history of use of Plague Vaccine U.S.P. are supportive and should be properly positioned for regulatory authorities, it should be kept in mind that so far as the new vaccine is concerned, it is the final formulation, delivered by the intranasal route, that must be safe and effective.

PharmaDerm Laboratories has indicated that all the components of the liposomes are pharmaceutical grade (either U.S.P. or other pharmacopoeial grade) and that the composition is "consistent with a well formulated dosage form and acceptable for dermal, transdermal, and mucosal applications". While for most pharmaceutical uses, liposomes would be considered "inactive", the current feeling with respect to vaccines is that liposomes act as adjuvants and as such are expected to modify the immune response rather than simply act as a delivery system. While new (*i.e.*, investigational) adjuvants would need to undergo substantial nonclinical evaluation as they would be classified as drugs or immunomodulators themselves, it is reassuring to hear that the PharmaDerm starting materials are well-characterized (it will be important to

incorporate such information in regulatory filings to support any data generated by the intranasal route). From our experience with liposomal formulations of anti-cancer drugs and from speaking with HPB and FDA regarding their views regarding liposome-formulated vaccines for intranasal administration, I can assure you that these materials are not a major concern to either agency with respect to safety, although they can be a major source of frustration with respect to manufacturing and stability of final formulations (see below). There is a need for confirmation of the general safety of the carrier system, but no particular concerns with liposome-based delivery systems, with the possible exception of aspiration.

Chemistry, Manufacturing, and Controls

It is perhaps prudent to discuss the expectations of regulatory authorities with respect to the chemistry and manufacturing of the new vaccine before reviewing the clinical and nonclinical studies that will be needed to support registration. The reason for this cannot be overstated as all lots of vaccine intended for investigational testing in humans must be prepared under GMP. As such, the characterization of the final formulation should be completed prior to the conduct of the clinical trials (or you run the risk of repeating the studies or completing additional studies to "bridge" into that database). While Plague Vaccine U.S.P., produced by Greer Laboratories, is presumably manufactured under GMP, the reformulation of this material into the liposomal formulation will be required to meet current Canadian GMPs. You should also note that HPB is in the process of revising its GMP guidelines to include an Annex for Schedule D (biologics) drugs. These are still draft, and will probably not be finalized and implemented for another year. As part of this, there will probably be the need to make reference in some way to the manufacturing data of Greer Laboratories (as a starting material). I am not sure if there is a Drug Master File for this product that can be referenced (with the manufacturer's permission), but this should be explored.

With respect to the manufacturing of the liposome-formulated product, I have enclosed an unpublished draft FDA guidance document for your consideration. It is entitled *Points to Consider in the Chemistry, Pharmacology, and Toxicology Development of Liposomal Drugs Intended for the Treatment of HIV Infection and Associated Disorders*. I would like to bring your attention to those areas of the document which relate to the chemistry, manufacturing, and controls of liposomal formulations, and confirm that these are the points which will be required to be addressed with respect to the liposomal vaccine.

While the clinical trials that will be required to support registration will be described below, you should be aware that in order to support an NDS, you will be required to provide data obtained

from the use of at least three (typically three to five) different lots of the final product to demonstrate and ensure consistency in the manufacturing process. During the IND process, however, you can perform investigations with data from only one lot. Similarly, it is our recommendation that animal safety studies (see below) be carried out with the same formulation(s) as those for human use, or they do not necessarily accurately provide a basis for assessing the nonclinical safety of the new product. As such, the need to develop consistent methods (and specifications) for the production of the liposomal formulation should be considered as a priority as it will certainly impact on the timing of the nonclinical and clinical studies.

Nonclinical Studies

Before any clinical trials are conducted, it will be expected that the safety of the formulation, by the intranasal route, be demonstrated in animals. Typically, nonclinical studies with new vaccines provide an assurance of safety, proof of concept, and information to determine a safe starting dose in humans.

It is our understanding that there are studies underway in mice investigating the efficacy (immunogenicity) of the liposome-formulated vaccine by the intranasal route. Ideally, these investigations should be conducted in compliance with Good Laboratory Practices (GLP) regulations. This is not an absolute requirement *per se*, but the level of comfort on the part of regulatory agencies when reviewing these data is increased if the data were generated under GLP or "GLP-like". These data will need to be included in any regulatory submissions as evidence of the nonclinical pharmacodynamics of the modified vaccine. As such, special emphasis should be placed on the quality of the study reports.

In addition to these immunogenicity studies, we feel that an acute toxicity study (*i.e.*, single dose) in a single species by the intranasal route should be performed with the liposomal formulation. As described, it is desirable to test the same material that is intended for human testing to accurately assess the safety. Because there will be a pathology assessment, such an investigation would need to be placed in a contract laboratory, and be conducted in accordance with GLP requirements.

It will be necessary to provide the rationale for the choice of species and also the dose selected in the safety study. Discussions with FDA have indicated that intranasal vaccines have been assessed in rabbits, rats, and/or guinea pigs. With respect to the modified plague vaccine, it may be beneficial to conduct such a study in mice, as there are many historical data with respect to the use of the mouse as a model to confirm the potency of plague vaccine (*e.g.*, U.S.P. Mouse

Potency Test). Despite its size, the mouse is used in toxicology as a model for assessing the administration of compounds by the intranasal and inhalation routes. For these reasons, as well as the ability to bridge into immunogenicity data being generated in the mouse with the modified vaccine, CanTox feels that the mouse would be an appropriate choice of species. A typical study design would incorporate the use of both sexes and 10 animals per sex per dose level. Depending on the study design of the immunogenicity studies your group is conducting, it may be possible to incorporate the toxicologic assessment into these investigations, as long as histopathologic assessment of the important organs and tissues from a sufficient number of animals, by a qualified individual, is included.

Regarding dose selection, it will be essential to assess the safety of a dose which provides an adequate margin of safety in relation to the human dose. This can be interpreted as anywhere from 10-20 times the dose of the final formulation on a mg/kg body weight (or mg/m^2 body surface area) basis. Unlike most acute toxicity studies (which look only at clinical signs and lethality), the study which we are proposing would need to incorporate histopathologic assessment of a number of tissues and organs, including, but not limited to, the nasal cavity (to assess local toxicity), the lungs (to assess any effects of aspiration), and draining lymph nodes. If toxic findings are observed, it may be necessary to further characterize the dose-response relationship of the findings.

CanTox does not feel that the registration of the product would require a more elaborate toxicology program (such as assessment of reproductive performance and teratogenicity, mutagenicity, or carcinogenicity).

Clinical Trials to Support Registration

As outlined earlier, all clinical studies need to be carried out under GCP regulations (which influences the cost and extent of documentation maintained), and before any investigative work in humans is allowed in Canada by a company, an IND must be filed with (and approved by) the BBR. Also, any work conducted in humans must be done with product manufactured in compliance with GMP regulations, as described earlier.

In general, the three phases of the clinical program for a new vaccine are:

I tolerability and immunogenicity established in small numbers of healthy adults (often up to 20 individuals);

II establishment of immunogenicity, safety, tolerability, dosage, dosage regimen, and final formulation. This phase can be broken down into 2 sub-phases:

IIa confirmation of immunogenicity, tolerability and optimization of dose (200-400 individuals over a 6-18 month period)

IIb definitive establishment of immunogenicity, tolerability and optimization of dosing regimen (500-1000 individuals over a 6-18 month period);

III establishing the protective efficacy of the vaccine in a blinded, placebo-controlled manner (not applicable in the case of a plague vaccine).

Clinical data (in healthy human volunteers) will be an absolute requirement to obtain regulatory approval of the modified plague vaccine in Canada (and elsewhere). As you are aware, the assessment of a vaccine for this indication precludes its assessment in well-controlled prospective studies. In fact, despite the fact that plague vaccines have been used since the late 19th century, their effectiveness has never been measured in such studies, and field experience (*i.e.*, reduction of the incidence and severity of disease resulting from the bites of infected rodent fleas) has been the basis for the use of the intramuscular vaccine in humans (supported by animal challenge studies). As such, "efficacy" for any modified plague vaccine will have to be based upon surrogate data that in effect allow you to bridge into the existing database for efficacy of intramuscular Plague Vaccine U.S.P.

In addition, it should be kept in mind that any claim that a sponsor wishes to make in the product labelling, package insert, *etc.*, must be supported by data and is subject to regulatory approval. The extent of the claim and the wording will depend entirely on the strength of the supporting data. In the case of the modified vaccine and modified immunization protocol (*i.e.*, intranasal administration of the liposomal formulation in addition to intramuscular injections of Plague Vaccine U.S.P.), it has been conveyed to CanTox that a claim such as "protects against the pneumonic form of *Yersinia pestis* infection in addition to the systemic form" would be desirable. As there can be no prospective clinical trials to test this hypothesis, it is highly unlikely that a regulatory body would allow such a definitive claim to be made. However, based upon the findings in animal challenge tests, altered immunity in humans (*e.g.*, increased pulmonary secretion of IgA and/or other non-invasive assessments), and correlations in animals between

enhanced protection against the pneumonic form of the disease and increased pulmonary secretion of IgA and increased lung cell immunity, it is felt that a strong claim could be made. For example, if the data support it, it may be possible to state something such as "because of the low incidence of plague disease, studies are not currently feasible to assess the effectiveness of the modified vaccine in protecting against the pneumonic form of *Yersinia pestis* infection in humans. However, data from animal studies and indirect evidence in healthy human volunteers supports the expectation that the modified vaccine will provide protection to most recipients against both the pneumonic and systemic forms of *Yersinia pestis* infection". We do not feel that the use of non-human primates in challenge tests, in addition to the mouse work which is underway, is warranted, unless there is reason to believe that non-human primates would be better models to assess the protective activity of the modified vaccine against the pneumonic form of the disease (keeping in mind that the appropriate immunization protocol in the species would need to be established).

The most practical and efficient way to demonstrate efficacy of a vaccine by a new route of administration (*i.e.*, where the formulation has not been changed) is to clearly demonstrate that, when compared to the original route of administration in a comparative study, the level of immunity (*e.g.*, the titres of antibodies to the same antigen and duration of conferred immunity) is the same. In such a situation, if the relationship between the development of antibodies to a specific antigen(s) and the prevention (or decreased severity) of a disease had been firmly established, then actual clinical efficacy studies would not be considered necessary to allow licensing of the product; this appears to be the case with plague vaccine antigens. In terms of the preclinical data which may be needed to support a clinical trial involving the use of the approved plague vaccine by the intranasal route (*i.e.*, in its present formulation at doses equal to or less than those in clinical use), CanTox does not feel that any formal animal toxicology studies *via* this route would be necessary. This conclusion is based upon the fact that the risks of the product by the more invasive, and bioavailable, intramuscular route have been well characterized, and that as part of the immunological characterization, there will be certain animal data generated *via* this route of administration. However, should unexpected adverse effects be observed in the animals treated by the intranasal route, then it may be necessary to characterize such findings in a more definitive safety study, as described for the re-formulated vaccine.

According to the directions for use of Plague Vaccine U.S.P., *Y. Pestis* contains at least 19 antigenic components. Five components (*i.e.*, Fraction 1 capsular antigen, the V and W antigens, exotoxin and endotoxin) correlate with immunogenic activity and virulence, whereas the remaining components have not been demonstrated to correlate with plague immunity or infection. Fraction 1 capsular antigen is the principal antigen involved with both virulence and immunity. Antibody

to Fraction 1 capsular antigen, determined by passive hemagglutination (PHA) test, correlates with protection against *Y. Pestis* infection in experimental animals; a comparable correlation between the titre for antibody to Fraction 1 capsular antigen (determined by PHA) and immunity appears to exist in humans. Although some experts consider a titre of antibody to Fraction 1 capsular antigen (determined by PHA) of at least 1:32 to be indicative of protection, the U.S. Public Health Service Immunization Practices Advisory Committee (ACIP) states that the minimum titre of antibody to Fraction 1 capsular antigen (determined by PHA) indicative of adequate protection against plague is 1:128 in animals.

The presumptive protective titre of antibody to Fraction 1 capsular antigen (1:128) is generally reached following intramuscular administration of an initial 1mL dose of Plague Vaccine followed by a second dose of 0.2mL. The titre increases following administration of a third dose of 0.2mL of vaccine, and persists for about 6-12 months. In one study in 29 adult male volunteers, following intramuscular administration of Plague Vaccine in doses of 1, 0.2 and 0.2mL on days 0, 90, and 270, respectively, the mean titre of antibody to Fraction 1 capsular, was 1:25, 1:140, and 1:576 at 15, 105, and 285 days respectively. However, in this study, 7% of the individuals failed to produce detectable antibodies even after the second booster dose. Thus individual variation in the response to vaccine does exist.

Another indirect method of assessing the ability of the modified vaccine to induce immunity in humans would be the utilization of the Mouse Protection Index (MPI). This method measures the capacity of serum antibodies from humans (and animals) to passively protect mice from virulent *Y. pestis*. While MPI scores can range from 0 to 33, an MPI value ≤10 generally is considered protective. Killed plague vaccines with acceptable immunogenic potency for mice stimulate the appearance of anti plague antibodies (MPI ≤ 10) in humans. The product monograph for Plague Vaccine U.S.P. reports that in monkeys and rats vaccinated with plague vaccine, 76% of animals possessing final PHA anti-F1 antibody titres ≥1:128 survived challenges with plague bacilli. Human subjects developing PHA anti-F1 antibody titres ≥1:128 generally had MPI values of ≤10. It is from the use of the MPI method as a means of determining human serum protective activity that Greer Laboratories demonstrated equivalence to the product produced by a previous manufacturer, Miles Inc. This study, conducted in 1993, utilized a single-blind design in adults with 19 or 20 individuals tested for each of three different lots of Greer Laboratories product. Results were expressed as mean geometric mean MPI values and the percent responding with an MPI ≤10 (*i.e.*, positive protection).

Based upon this information, we believe that there are sufficient data to justify the use of the MPI and the measurement of titres to antibody to Fraction 1 capsular antigen as endpoints from which

to assess the "efficacy" of the intranasal modified vaccine in clinical trials. Because there would be expected to be differences between the induction of systemic immunity (as is produced by intramuscular injection of the plague vaccine) and immunity induced against the pneumonic form of the disease (as is proposed for the intranasal delivery of the modified vaccine), some work may have to be done to establish and justify, for example, a modified MPI value to take into account these differences. As pointed out in your letter to CanTox of July 3, 1997, the currently licenced vaccine is not particularly effective in protecting against the pneumonic form of plague, indicating that serum antibody titres and/or human serum protective activity are not the only consideration. This presents some difficulty as the existing database for the systemic form of the disease is quite clear as to what is considered "protective" in humans. With respect to safety and efficacy in humans, the fact that the intranasal formulation will be administered in addition to the intramuscular injections means that the design of any clinical investigation must consider two important endpoints: (1) the effect of the intranasal dose of modified vaccine on the type and severity of adverse effects experienced with intramuscular injections, and (2) the effect of the intranasal dose of modified vaccine on the level of systemic immunity induced by the intramuscular injections (*i.e.*, demonstration that systemic immunity is not decreased through interference).

While it is not within the scope of this assessment to devise the actual study protocol, it is possible to obtain approval based upon the results of one well conducted study. Given that an NDS would require clinical data generated from the use of at least three different lots (to demonstrate and ensure consistency in the manufacturing process), and that for statistical reasons, a minimum of 100 individuals would need to be included for each lot used, any combined clinical study with would require at least 300 individuals, covering both sexes and the ages for which the modified vaccine would be recommended for.

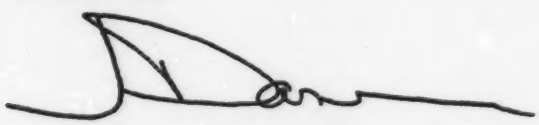
Post Approval Considerations

Currently, 99% of all biological products approved for use in Canada must undergo lot release. This means that the manufacturer must test each and every lot of a product before it is released for sale in order to confirm potency, *etc.* In the case of Plague Vaccine U.S.P., potency is confirmed using the Specific Mouse Protection Test in which immunized animals are challenged with U.S. Reference Plague Vaccine (as described in the U.S. Pharmacopoeia). At the same time as the company is conforming to its lot release requirements, it is typically required to submit samples to the BBR for analysis (unless the product in question has been specifically exempted from this requirement).

Another major requirement for approved products is post marketing surveillance. Companies are required to maintain detailed files of Adverse Drug reactions (ADRs) and these data periodically are provided to the BBR, usually within the annual report to the Bureau. "Serious" ADRs require more expedited reporting, usually with 7 to 15 days of the incident being reported to the company.

I trust that this information addresses your needs with respect to identifying the regulatory steps required to gain approval of this product and data which will be needed to support registration. Please let me know if you have any questions related to the contents of this letter.

Yours sincerely,
CanTox Inc.



Jon Daniels, Ph.D., DABT
Senior Scientist

Enclosure

Research Report – Appendix B to Annex A, Part II

Protective Capacity of *Y. pestis* Antigens

II. Introduction

From results presented at the tri-National plague meeting in 1998, it was clear that recombinant V antigen of *Y. pestis* was an excellent choice for a subunit vaccine which would protect against both pneumonic and bubonic plague. This is in contrast to the pH6 antigen which was not effective as a monomeric subunit. The V antigen is released from the *Y. pestis* cell surface *in vivo* and can exist in both monomeric and multimeric forms. Its role in pathogenesis is not clear at present, but it has been shown to have immunomodulatory function and may act as an aggressin. Therefore, it was of interest to test the protective capacity of recombinant V antigen following a combination of subcutaneous and intranasal administration. This antigen has already been shown to protect mice after injection and our hypothesis was that protection would be enhanced by formulating the antigen with liposomes and delivering intranasally.

III. Materials and Methods

1. Preparation of recombinant V antigen.

The V antigen was prepared from recombinant *E. coli* cells by passage through a nickel-chelated NTA resin as described in the Materials and Methods section of the Research Report for Annex A. Briefly, bacteria were grown to mid log phase and V antigen production was induced by the addition of IPTG. The protein was expressed in a soluble fashion with a six amino acid histidine tag. Following harvest of the bacteria, they were lyzed by sonication and the lysate mixed with nickel-chelated NTA resin. This was incubated for one hour at 4°C with gentle mixing. The slurry was then loaded into a 1 cm diameter column and washed six times with Buffer. The antigen was eluted with a buffer containing imidazole and dialyzed against phosphate-buffered saline.

2. *Y. pestis* challenge.

Y. pestis strain GB was obtained from the Chemical and Biological Defense Establishment, Porton Down, UK and was stored on "Protect" Bacterial Preserver Beads (TSC Limited, Haywood, UK). One to two beads were transferred to 10 ml of heart infusion broth and grown overnight at 28°C. This culture was diluted 10-fold into fresh heart infusion broth and grown overnight in a side-arm flask to an OD of 0.4, corresponding to approximately 3×10^8 colony-forming units of bacteria. Animals were anesthetized by Metaphane inhalation and 25 µl of *Y. pestis* containing approximately 6100 CFU of bacteria was applied to the nares of each mouse. Animals were monitored closely for signs of disease and distress.

3. Vaccination studies.

Female mice at 6 weeks of age (CF7B1/6) were immunized in groups of twelve. The first immunization was delivered subcutaneously followed twenty-one days later by a booster delivered intranasally as described in Appendix A to Annex A. Thirty-one days after immunization mice were challenged intranasally with *Y. pestis* as described above.

4. Statistical Analyses.

All data was analyzed as described in Part II of Annex A.

IV. Results and Discussion

1. Preparation of recombinant V antigen.

Approximately 10 mg of V antigen was prepared from *E. coli* by affinity chromatography as described above. This material was estimated to be in excess of 95% purity by polyacrylamide gel electrophoresis (Figure B-1). This material was also used to establish immunological assays to measure the response following immunization. In this case, we used an enzyme-linked immunosorbent assay in which 10 nanograms of recombinant V antigen was used to coat Immulon II 96 well plates. The procedure is described in detail in Part II of the Annex A Research Report.

2. *Y. pestis* aerosol challenge model.

All experiments involving the use of *Y. pestis* were carried out at the Defense Research Establishment, Suffield. The 50% lethal dose (LD50) was calculated as described in Part II of the Annex A Research Report. The LD50 was found to be approximately 600 CFU. All animals which were infected showed ruffled fur, hunched backs and were generally lethargic in response to external stimuli. These mice were considered moribund and were euthanized humanely in accordance with the guidelines of the Canadian Council on Animal Care. For vaccination studies described below, a dose of 10 LD50 of *Y. pestis* strain GB (6.1×10^3 CFU) was delivered to the nares of mice as described above.

3. Protective capacity of V antigen

Following subcutaneous and intranasal vaccination with 10 μg of V antigen formulated in liposomes, all animals showed an excellent immune response as shown in Figure B-2A. This immune response was greater than the V-specific response of any of the mice immunized with the conventional plague vaccine (report on Annex A). However, these mice were not significantly protected against experimental challenge following intranasal administration of virulent *Y. pestis* (Figure B-2B). The reason for this lack of protection is unclear since workers in the USA and UK have demonstrated that injection of V antigen adjuvanted with aluminum hydroxide results in protection against the pneumonic form of the disease. It does suggest that the lack of protection which we observed following mucosal immunization with the *Escherichia coli* and human adenovirus-vectored vaccines may have been due to the form of the antigen as opposed to the vector system used.

Given the excellent immune response to the antigen, we assume that the dose employed (10 μg) was suitable for this experiment. However, as was mentioned above, V antigen can exist in monomeric and multimeric forms and it is possible that the monomeric form of the antigen is not protective when delivered intranasally. Also, it is possible that intranasal administration with recombinant V antigen does not induce the appropriate type of immune response in the lungs. The data presented at the Tri-National Plague Meeting certainly suggests that there is a direct correlation between V antigen-specific IgG titres, but this has not been extended to include IgA. The lack of protection is in contrast to the results observed when mice were immunized in an identical fashion with a vaccine containing the conventional plague vaccine formulated with liposomes. In the latter case, the liposome formulation significantly enhanced immunogenicity following intranasal delivery, resulting in enhanced protection against experimental challenge. Although most researchers have used specific antibody levels as a measure of immunity to *Y. pestis*, it is clear that as with most intracellular pathogens, the cell-mediated response following immunization is crucial for full protection to be obtained. It would be of interest to further characterize immune responses to V antigen following formulation in liposomes in a similar fashion to that described for the conventional vaccine in the report on Appendix A to Annex A.

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Figure B-2. Humoral anti-V titres after immunization with V antigen incorporated Into liposomes and mortality following *Y. pestis* challenge..... 74

Figure B-1. SDS-PAGE of recombinant V antigen.

The protein was prepared by affinity chromatography as described in the text. The purity of the antigen was estimated to be in excess of 95%.

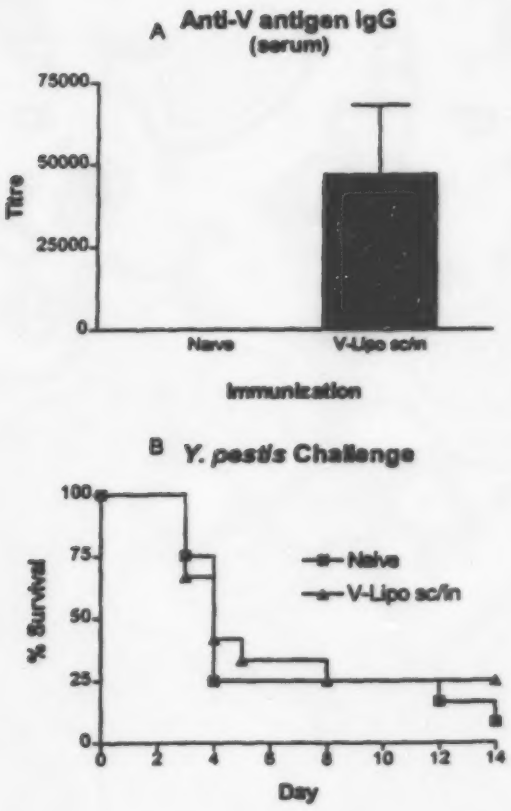

Figure B-2. Humoral anti-V titres after immunization with V antigen incorporated into liposomes and mortality following *Y. pestis* challenge.

(A) Systemic humoral immune responses, and; (B) Protection against *Y. pestis* challenge. Mice were immunized with 10 μg of recombinant V antigen formulated in liposomes. Animals received a primary immunization with V antigen by the subcutaneous (s.c.) route and 21 days later were boosted intranasally (i.n.). The anti-V antigen IgG titres were determined in serum. Antibody titres are expressed as the mean titre ± SEM of 5 individual mice. Two weeks after the second immunization all animals were challenge with 10 LD_{50} of *Y. pestis*.